(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 September 2001 (20.09.2001)

PCT

(10) International Publication Number
WO 01/69261 A2

(51) International Patent Classification[7]: G01N 33/68, C07K 14/47, A61P 9/10, 25/28, A61K 38/17

(21) International Application Number: PCT/GB01/01106

(22) International Filing Date: 14 March 2001 (14.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0006285.1	15 March 2000 (15.03.2000)	GB
0028734.2	24 November 2000 (24.11.2000)	GB
09/724,391	28 November 2000 (28.11.2000)	US

(71) Applicant *(for all designated States except US)*: **OXFORD GLYCOSCIENCES (UK) LTD.** [GB/GB]; The Forum, 86 Milton Park, Abingdon, Oxon OX14 4RY (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HERATH, Herath, Mudiyanselage, Athula, Chandrasiri** [GB/GB]; Oxford GlycoSciences (UK) Ltd., The Forum, 86 Milton Park, Abingdon, Oxon OX14 4RY (GB). **PAREKH, Rajesh, Bhikhu** [GB/GB]; Oxford GlycoSciences (UK) Ltd., The Forum, 86 Milton Park, Abingdon, Oxon OX14 4RY (GB). **ROHLFF, Christian** [DE/GB]; Oxford GlycoSciences (UK) Ltd., The Forum, 86 Milton Park, Abingdon Oxon OX14 4RY (GB).

(74) Agents: **LEE, Nicholas, John** et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/69261 A2

(54) Title: PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF VASCULAR DEMENTIA

(57) Abstract: The present invention provides methods and compositions for screening, diagnosis and prognosis of Vascular Dementia, for monitoring the effectiveness of Vascular Dementia treatment, identifying patients most likely to respond to a particular therapeutic treatment and for drug development. Vascular Dementia-Associated Features (VFs), detectable by two-dimensional electrophoresis of cerebrospinal fluid, serum or plasma are described. The invention further provides Vascular Dementia-Associated Protein Isoforms (VPIs) detectable in cerebrospinal fluid, serum or plasma, preparations comprising isolated VPIs, antibodies immunospecific for VPIs, and kits comprising the aforesaid.

PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF VASCULAR DEMENTIA

1. INTRODUCTION

The present invention relates to the identification of proteins and protein isoforms that are associated with Vascular Dementia and its onset and development, and of genes encoding the same, and to their use for *e.g.*, clinical screening, diagnosis, prognosis, therapy and prophylaxis, as well as for drug screening and drug development.

2. BACKGROUND OF THE INVENTION

Vascular dementia is the second most common cause of dementia in the US and Europe and a very heterogeneous disease with many factors contributing to the overall pathogenesis. Eight major types of vascular dementia have been identified: 1. Multi-infarct dementia secondary to large cerebral emboli, 2. Strategically placed infarctions causing dementia, 3. Multiple subcortical lacunar lesions secondary to atherosclerosis or degenerative arteriolar changes, 4. Binswanger's disease (arteriosclerotic subcortical leukoencephalopathy), 5. Mixtures of types 1, 2 and 3, 6. Haemorrhagic lesions causing dementia, 7. Subcortical dementia secondary to hereditary factors, and 8. Mixtures of dementia of the Alzheimer's type and vascular dementia (Konno et al. *Drugs Aging* (1997) 11:361-73). A great need exists for an improved diagnosis of vascular dementia. Today, the clinician depends on clinical examinations, the patient's history, and, possibly, brain imaging to recognize signs of vascular dementia such as cerebrovascular damage. Currently diagnosis combines several methods including brain imaging of the injured site via computed tomography (CT) or magnetic resonance imaging (MRI) (Kistler et al. *Stroke* (1984) 15:417-26), duplex and transcranial Doppler methods (Comerota et al. *Surgery* (1981) 6:718-29),and positron emission tomography (Frackowiak and Kjaellman *Neurol Clin North Am* (1983) 1:183-200).

Numerous clinical criteria are used in the diagnosis of vascular dementia VD, resulting in variations the frequency of VD diagnosis depending on the applied criteria (4 clinical definitions of VD are currently used: the Hachinski Ischemic Score (HIS), the Alzheimer Disease Diagnostic and Treatment Centers (ADDTC), National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l' Enseignement en Neurosciences (NINDS-AIREN), and Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)) (Chui et al. *Arch Neurol* (2000) 57:191-6). Even though standardized neuropsychological assessments facilitate the differential diagnosis of

vascular dementia from Lewy bodies dementia and Alzheimer's disease (Ballard et al. *Dement Geriatr Cogn Disord* (1999) 10:104-8), diagnostic criteria for vascular dementia still require validation (Nyenhuis and Gorelick *J Am Geriatr Soc* (1998) 46:1437-48).

In the majority of neurological disorders like vascular dementias, little is known about a link between changes at a cellular and/or molecular level and nervous system structure and function. In an acute stroke a quick assessment of the cause, severity and chance of progression or recurrence is necessary to ensure an optimal treatment to stabilize or reverse the effects. Few biochemical changes have been identified in vascular dementia. Consequently the identification and characterization of cellular and/or molecular causative defects and neuropathologies are necessary for improved treatment of vascular dementia associated disorders. Due to the possibility of worsening or recurrence, speedy diagnosis would be of great benefit, in particular to categorize the patient as follows:

1. Stroke versus nonstroke such as cerebral tumors and subdural hematoma
2. Hemorrhage versus infraction
3. Specific pathophysiological subtypes of cerebral infarction (Donnan *Lancet* (1992) 339:473).

In particular cerebrovascular dementia often coexists with other causes of dementia (Erkinjuntti *Int Psychogeriatr* (1997) 9 Suppl 1:51-8; discussion 77-83) complicating a proper diagnosis and effective treatment strategies. The majority of vascular dementias are caused by both genetic and environmental factors (Plassman and Breitner *J Am Geriatr Soc* (1996) 44:1242-50), although an increased prevalence of vascular dementia has been demonstrated in the cerebral arteriopathy syndrome, a genetic form of vascular dementia (Salloway and Hong *J Geriatr Psychiatry Neurol* (1998) 11:71-7), apolipoprotein E gene polymorphism in Binswanger's disease and vascular dementia (Higuchi et al. *Clin Genet* (1996) 50:459-61) and hereditary cystatin C amyloid angiopathy (HCCAA) in Icelandic patients and hereditary cerebral haemorrhage with amyloidosis, Dutch type (HCHWA-D) (Wang et al. APMIS (1997) 105:41-7).

Although genetics and genotyping may help to define the heritable risk for Vascular dementia, the utility for diagnosis, prognosis and treatment of vascular dementia may be considerably less. Furthermore, no CNS tissue necessary for any gene expression analysis can be obtained for a living patient under normal circumstances. Proteomic approaches appear most suitable for a molecular dissection of such disease phenotypes in the central nervous system (CNS). The entire CNS is largely inaccessible to meaningful mRNA expression-based analyses of primary human material, since post mortem delays in primary human brain tissue

affects mRNAs more readily than proteins (Edgar et al. *Molecular Psychiatry* (1999) 4:173-17). Given that the CSF bathes the brain, changes in its protein composition may reveal alterations in CNS protein expression pattern causatively or diagnostically linked to the disease. Reasonable amounts of DAPs are secreted or released into body fluids by diseased tissue in the living patient at the onset and/or during progression of the disease. In many cases these alterations will be independent of the genetic makeup of the individual and rather directly related to a set of molecular and cellular alterations contribution to the pathogenic phenotype (Carpenter *J Psychiatr Res* (1998) 32:191-5).

Current treatments of vascular dementia include antithrombic therapies (Crowth and Ginsberg in Stroke, Pathophysiology, Diagnosis, and Management Eds. Barnett, Mohr et al. Year, Churchill Livingston, a division of Harcourt Brace & Company), thrombolytic and defibrinogenating agents (Brott and Hacke in Stroke, supra), antiplatelet agents (Weksler in Stroke, supra) and neuroprotective agents (Gluckmann and Gunn in Neuroprotection in CNS diseases, Eds. Baer and Beal Year, Marcel Dekker, Inc. New York).

Currently vascular dementia has no objective biochemical markers useful for diagnosis and prognosis in living patients. The identification of disease associated proteins (DAPs) in the CSF of vascular dementia patients may provide important insights into disease pathology and opportunities for better diagnosis and treatment strategies. However, currently the majority of DAPs identified in vascular dementia also occur in other diseases, such as the CSF tau protein, CSF neuron-specific enolase and CSF neurofilament light protein (Wallin et al. *Alzheimer Dis Assoc Disord* (1999) 13 Suppl 3, S102-5). Several autopsy series of patients with dementia suggest that a high percentage of patients clinically diagnosed with vascular dementia at autopsy will also be found to have pathological changes consistent with coexisting Alzheimer's disease (Nolan et al. *J Am Geriatr Soc* (1998) 46:597-604; Snowdon et al. JAMA (1997) 277:813-817; Hulette et al. *Neurol* (1997) 48:668-672). Therefore in many cases, differential diagnosis of VD to other dementias, such as Alzheimer's disease, Lewy body dementia remains difficult. Therefore, the specificity and the sensitivity of distinguishing individual neurological disorders as well as acute and chronic CNS disease may require the selection of a repertoire of DAPs rather than an individual protein.

Due to the high rates at which other disorders co-occur with Vascular dementia, the time consuming nature of existing, largely inadequate, tests and their expense it would be highly desirable to measure a substance or substances in samples of cerebrospinal fluid (CSF), blood or urine that would lead to a positive diagnosis of vascular dementia or that would help to exclude vascular dementia from the differential diagnosis.

Therefore, a need exists to identify sensitive and specific biomarkers for the diagnosis, to assess severity and predict the outcome of vascular dementia in living subjects. Additionally, there is a clear need for new therapeutic agents for vascular dementia that work quickly, potently, specifically, and with fewer side effects.

3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for clinical screening, diagnosis, prognosis, therapy and prophylaxis of Vascular Dementia, for monitoring the effectiveness of Vascular Dementia treatment, for selecting participants in clinical trials, for identifying patients most likely to respond to a particular therapeutic treatment and for screening and development of drugs for treatment of Vascular Dementia.

A first aspect of the invention provides methods for diagnosis of Vascular Dementia that comprise analyzing a sample of cerebrospinal fluid (CSF) by two-dimensional electrophoresis to detect the presence or level of at least one Vascular Dementia-Associated Feature (VF), *e.g.*, one or more of the VFs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, for drug screening and development, and identification of new targets for drug treatment.

A second aspect of the invention provides methods for diagnosis of Vascular Dementia that comprise detecting in a sample of CSF the presence or level of at least one Vascular Dementia-Associated Protein Isoform (VPI), *e.g.*, one or more of the VPIs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, drug screening and development, and identification of new targets for drug treatment.

A third aspect of the invention provides antibodies, *e.g.* monoclonal and polyclonal antibodies capable of immunospecific binding to a VPI, *e.g.*, a VPI disclosed herein.

A fourth aspect of the invention provides a preparation comprising an isolated VPI, *i.e.*, a VPI free from proteins or protein isoforms having a significantly different isoelectric point or a significantly different apparent molecular weight from the VPI.

A fifth aspect of the invention provides methods of treating Vascular Dementia, comprising administering to a subject a therapeutically effective amount of an agent that modulates (*e.g.*, upregulates or downregulates) the expression or activity (*e.g.* enzymatic or binding activity), or both, of a VPI in subjects having Vascular Dementia, in order to prevent

or delay the onset or development of Vascular Dementia, to prevent or delay the progression of Vascular Dementia, or to ameliorate the symptoms of Vascular Dementia.

A sixth aspect of the invention provides methods of screening for agents that modulate (*e.g.*, upregulate or downregulate) a characteristic of, *e.g.*, the expression or the enzymatic or binding activity, of a VPI, a VPI analog, or a VPI-related polypeptide.

3.1. Definitions

The term "VPI analog" as used herein refers to a polypeptide that possesses a similar or identical function as a VPI but need not necessarily comprise an amino acid sequence that is similar or identical to the amino acid sequence of the VPI, or possess a structure that is similar or identical to that of the VPI. As used herein, an amino acid sequence of a polypeptide is "similar" to that of a VPI if it satisfies at least one of the following criteria: (a) the polypeptide has an amino acid sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the amino acid sequence of the VPI; (b) the polypeptide is encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding at least 5 amino acid residues (more preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues) of the VPI; or (c) the polypeptide is encoded by a nucleotide sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the nucleotide sequence encoding the VPI. As used herein, a polypeptide with "similar structure" to that of a VPI refers to a polypeptide that has a similar secondary, tertiary or quarternary structure as that of the VPI. The structure of a polypeptide can determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

The term "VPI fusion protein" as used herein refers to a polypeptide that comprises (i) an amino acid sequence of a VPI, a VPI fragment, a VPI-related polypeptide or a fragment of a VPI-related polypeptide and (ii) an amino acid sequence of a heterologous polypeptide (*i.e.*, a non-VPI, non-VPI fragment or non-VPI- related polypeptide).

The term "VPI homolog" as used herein refers to a polypeptide that comprises an amino acid sequence similar to that of a VPI but does not necessarily possess a similar or identical function as the VPI.

The term "VPI ortholog" as used herein refers to a non-human polypeptide that (i) comprises an amino acid sequence similar to that of a VPI and (ii) possesses a similar or identical function to that of the VPI.

The term "VPI-related polypeptide" as used herein refers to a VPI homolog, a VPI analog, an isoform of VPI, a VPI ortholog, or any combination thereof.

The term "derivative" as used herein refers to a polypeptide that comprises an amino acid sequence of a second polypeptide which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The derivative polypeptide possess a similar or identical function as the second polypeptide.

The term "fragment" as used herein refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, or at least 250 amino acid residues) of the amino acid sequence of a second polypeptide. The fragment of a VPI may or may not possess a functional activity of the second polypeptide.

The term "fold change" includes "fold increase" and "fold decrease" and refers to the relative increase or decrease in abundance of a VF or the relative increase or decrease in expression or activity of a polypeptide (*e.g.* a VPI) in a first sample or sample set compared to a second sample (or sample set). A VF or polypeptide fold change may be measured by any technique known to those of skill in the art, however the observed increase or decrease will vary depending upon the technique used. Preferably, fold change is determined herein as described in the Examples *infra*.

The term "isoform" as used herein refers to variants of a polypeptide that are encoded by the same gene, but that differ in their pI or MW, or both. Such isoforms can differ in their amino acid composition (*e.g.* as a result of alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation). As used herein, the term "isoform" also refers to a protein that exists in only a single form, *i.e.*, it is not expressed as several variants.

The term "modulate" when used herein in reference to expression or activity of a VPI or ε VPI-related polypeptide refers to the upregulation or downregulation of the expression or activity of the VPI or a VPI-related polypeptide. Based on the present disclosure, such modulation can be determined by assays known to those of skill in the art or described herein.

The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (*i.e.*, % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci. USA* (1990) 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. The NBLAST and XBLAST programs of Altschul et al, *J. Mol. Biol.* (1990) 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al, *Nucleic Acids Res.* (1997) 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti *Comput. Appl. Biosci.* (1994) 10:3-5; and FASTA described in Pearson and Lipman *Proc. Natl. Acad. Sci. USA* (1988)

85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an image obtained from 2-dimensional electrophoresis of human CSF, which has been annotated to identify twelve landmark features, designated CSF1 to CSF12.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention described in detail below provides methods and compositions for clinical screening, diagnosis and prognosis of Vascular Dementia in a mammalian subject for identifying patients most likely to respond to a particular therapeutic treatment, for monitoring the results of Vascular Dementia therapy, for drug screening and drug development. The invention also encompasses the administration of therapeutic compositions to a mammalian subject to treat or prevent Vascular Dementia. The mammalian subject may be a non-human mammal, but is preferably human, more preferably a human adult, *i.e.* a human subject at least 21 (more preferably at least 35, at least 50, at least 60, at least 70, or at least 80) years old. For clarity of disclosure, and not by way of limitation, the invention will be described with respect to the analysis of CSF samples. However, as one skilled in the art will appreciate, the assays and techniques described below can be applied to other types of samples, including a body fluid (*e.g.* blood, serum, plasma, saliva or urine), a tissue sample from a subject at risk of having or developing Vascular Dementia (*e.g.* a biopsy such as a brain biopsy) or homogenate thereof. The methods and compositions of the present invention are useful for screening, diagnosis and prognosis of a living subject, but may also be used for postmortem diagnosis in a subject, for example, to identify family members of the subject who are at risk of developing the same disease.

As used herein, cerebrospinal fluid (CSF) refers to the fluid that surrounds the bulk of the central nervous system, as described in Physiological Basis of Medical Practice (J.B. West, ed., Williams and Wilkins, Baltimore, MD 1985). CSF includes ventricular CSF and lumbar CSF. As used herein, the term "serum" refers to the supernatant fluid produced by clotting and centrifugal sedimentation of a blood sample. As used herein, the term "plasma" refers to the supernatant fluid produced by inhibition of clotting (for example, by citrate or EDTA) and centrifugal sedimentation of a blood sample. The term "blood" as used herein includes serum and plasma.

5.1 Vascular Dementia-Associated Features (VFs)

In one aspect of the invention, two-dimensional electrophoresis is used to analyze CSF from a subject, preferably a living subject, in order to detect or quantify the expression of one or more Vascular Dementia-Associated Features (VFs) for screening, prevention or diagnosis of Vascular Dementia, to determine the prognosis of a subject having Vascular Dementia, to monitor progression of Vascular Dementia, to monitor the effectiveness of Vascular Dementia therapy, for identifying patients most likely to respond to a particular therapeutic treatment, or for drug development. As used herein, "two-dimensional electrophoresis" (2D-electrophoresis) means a technique comprising isoelectric focusing, followed by denaturing electrophoresis; this generates a two- dimensional gel (2D-gel) containing a plurality of separated proteins. Preferably, the step of denaturing electrophoresis uses polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Especially preferred are the highly accurate and automatable methods and apparatus ("the Preferred Technology") described in International Application No. 97GB3307 (published as WO 98/23950) and in U.S. Application No. 08/980,574, both filed December 1, 1997, each of which is incorporated herein by reference in its entirety with particular reference to the protocol at pages 23-35. Briefly, the Preferred Technology provides efficient, computer-assisted methods and apparatus for identifying, selecting and characterizing biomolecules (*e.g.* proteins, including glycoproteins) in a biological sample. A two-dimensional array is generated by separating biomolecules on a two-dimensional gel according to their electrophoretic mobility and isoelectric point. A computer-generated digital profile of the array is generated, representing the identity, apparent molecular weight, isoelectric point, and relative abundance of a plurality of biomolecules detected in the two-dimensional array, thereby permitting computer-mediated comparison of profiles from multiple biological samples, as well as computer aided excision of separated proteins of interest.

A preferred scanner for detecting fluorescently labelled proteins is described in WO 96/36882 and in the Ph.D. thesis of David A. Basiji, entitled "Development of a High-throughput Fluorescence Scanner Employing Internal Reflection Optics and Phase-sensitive Detection (Total Internal Reflection, Electrophoresis)", University of Washington (1997), Volume 58/12-B of Dissertation Abstracts International, page 6686, the contents of each of which are incorporated herein by reference. These documents describe an image scanner designed specifically for automated, integrated operation at high speeds. The scanner can image gels that have been stained with fluorescent dyes or silver stains, as well as storage phosphor screens. The Basiji thesis provides a phase-sensitive detection system for

discriminating modulated fluorescence from baseline noise due to laser scatter or homogeneous fluorescence, but the scanner can also be operated in a non-phase-sensitive mode. This phase-sensitive detection capability would increase the sensitivity of the instrument by an order of magnitude or more compared to conventional fluorescence imaging systems. The increased sensitivity would reduce the sample-preparation load on the upstream instruments while the enhanced image quality simplifies image analysis downstream in the process.

A more highly preferred scanner is the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK), which is a modified version of the above described scanner. In the Apollo 2 scanner, the gel is transported through the scanner on a precision lead-screw drive system. This is preferable to laying the glass plate on the belt-driven system that is described in the Basiji thesis, as it provides a reproducible means of accurately transporting the gel past the imaging optics.

In the Apollo 2 scanner, the gel is secured against three alignment stops that rigidly hold the glass plate in a known position. By doing this in conjunction with the above precision transport system, the absolute position of the gel can be predicted and recorded. This ensures that co-ordinates of each feature on the gel can be determined more accurately and communicated, if desired, to a cutting robot for excision of the feature. In the Apollo 2 scanner, the carrier that holds the gel has four integral fluorescent markers for use to correct the image geometry. These markers are a quality control feature that confirms that the scanning has been performed correctly.

In comparison to the scanner described in the Basiji thesis, the optical components of the Apollo 2 scanner have been inverted. In the Apollo 2 scanner, the laser, mirror, waveguide and other optical components are above the glass plate being scanned. The scanner described in the Basiji thesis has these components underneath. In the Apollo 2 scanner, the glass plate is mounted onto the scanner gel side down, so that the optical path remains through the glass plate. By doing this, any particles of gel that may break away from the glass plate will fall onto the base of the instrument rather than into the optics. This does not affect the functionality of the system, but increases its reliability.

Still more preferred is the Apollo 3 scanner, in which the signal output is digitized to the full 16-bit data without any peak saturation or without square root encoding of the signal. A compensation algorithm has also been applied to correct for any variation in detection sensitivity along the path of the scanning beam. This variation is due to anomalies in the optics and differences in collection efficiency across the waveguide. A calibration is

performed using a perspex plate with an even fluorescence throughout. The data received from a scan of this plate are used to determine the multiplication factors needed to increase the signal from each pixel level to a target level. These factors are then used in subsequent scans of gels to remove any internal optical variations.

As used herein, the term "feature" refers to a spot detected in a 2D gel, and the term "Vascular Dementia-Associated Feature" (VF) refers to a feature that is differentially present in a sample (*e.g.* a sample of CSF) from a subject having Vascular Dementia compared with a sample (*e.g.* a sample of CSF) from a subject free from Vascular Dementia. As used herein, a feature (or a protein isoform of VPI, as defined *infra*) is "differentially present" in a first sample with respect to a second sample when a method for detecting the feature, isoform or VPI (*e.g.*, 2D electrophoresis or an immunoassay) gives a different signal when applied to the first and second samples. A feature, isoform or VPI is "increased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or VPI is more abundant in the first sample than in the second sample, or if the feature, isoform or VPI is detectable in the first sample and undetectable in the second sample. Conversely, a feature, isoform or VPI is "decreased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or VPI is less abundant in the first sample than in the second sample or if the feature, isoform or VPI is undetectable in the first sample and detectable in the second sample.

Preferably, the relative abundance of a feature in two samples is determined in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, *e.g.*, (a) to the total protein in the sample being analyzed (*e.g.*, total protein loaded onto a gel); (b) to an Expression Reference Feature (ERF) *i.e.*, a feature whose abundance is invariant, within the limits of variability of the Preferred Technology, in the population of subjects being examined, *e.g.* the ERFs disclosed below, or (c) more preferably to the total signal detected from all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

The VFs disclosed herein have been identified by comparing CSF samples from subjects having Vascular Dementia against CSF samples from subjects free from Vascular Dementia. Subjects free from Vascular Dementia include subjects with no known disease or

condition (normal subjects) and subjects with diseases (including neurological and neurodegenerative diseases) other than Vascular Dementia.

Two groups of VFs have been identified through the methods and apparatus of the Preferred Technology. The first group consists of VFs that are decreased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia. These VFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table I.

Table I. VFs Decreased in CSF of Subjects Having Vascular Dementia

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-4	8.89	11749	>100	
VF-5	9.16	20681	>100	
VF-12	7.62	70511	58.28	
VF-13	6.18	105482	34.78	
VF-14	4.65	102603	16.85	
VF-15	5.73	14776	30.68	
VF-16	4.65	32509	8.11	
VF-17	9.50	13985	10.15	
VF-18	6.45	94205	6.52	
VF-19	8.54	54625	5.28	
VF-20	6.30	73287	5.58	
VF-21	6.35	164223	4.70	
VF-22	9.04	11790	16.20	0.03689
VF-23	6.58	93680	4.60	
VF-24	7.51	37524	4.83	
VF-25	9.32	13044	8.57	
VF-26	9.18	48532	4.24	
VF-27	9.76	13546	7.04	
VF-29	5.26	11388	7.37	
VF-30	4.65	10120	4.15	
VF-31	7.80	29113	3.80	
VF-32	6.92	109447	7.33	
VF-33	4.34	10961	8.73	
VF-34	4.12	63272	7.14	
VF-35	5.65	13084	6.84	
VF-36	8.99	61111	5.20	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-37	9.80	23795	4.30	
VF-38	8.79	22458	6.43	
VF-41	4.91	38741	3.28	0.01219
VF-42	6.15	25232	2.74	0.03671
VF-43	5.67	48092	2.74	0.01219
VF-44	5.65	150534	2.09	0.03689
VF-45	4.53	11037	2.11	0.03734
VF-46	4.86	38741	2.61	0.01219
VF-47	5.49	57515	2.65	0.01996
VF-48	4.99	15072	2.24	0.02157
VF-50	5.48	55124	2.43	0.01219
VF-51	6.53	75865	2.01	0.03734
VF-52	5.37	123390	2.14	0.03671
VF-53	6.61	72071	1.82	0.03671
VF-54	4.57	13499	1.98	0.03671
VF-55	5.30	49423	1.94	0.01219
VF-57	9.18	39998	1.61	0.03671
VF-58	4.86	135312	1.95	0.03671
VF-60	5.66	21021	1.49	0.02157
VF-64	5.77	52354	1.45	0.01219
VF-66	5.01	25963	1.47	0.03671
VF-68	5.66	63184	1.46	0.01219
VF-171	8.40	14528	53.08	
VF-172	9.05	18350	41.40	
VF-173	4.19	34949	36.28	
VF-174	5.29	86159	28.38	
VF-175	4.83	184426	21.03	
VF-176	4.17	34311	18.79	
VF-177	4.51	50790	14.64	
VF-178	4.66	12080	14.51	
VF-179	9.19	37198	12.72	
VF-180	9.12	38200	12.34	
VF-181	9.65	65654	11.58	
VF-182	4.76	185225	11.13	
VF-183	4.58	52595	10.72	
VF-184	5.32	65751	10.07	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-185	4.39	89591	8.81	
VF-186	9.16	33591	8.80	
VF-187	6.87	66392	8.80	
VF-188	7.89	29058	7.13	
VF-189	8.54	34332	6.99	
VF-190	7.92	77836	6.73	
VF-191	6.27	186027	5.77	
VF-192	9.62	88830	5.64	
VF-193	9.82	67749	5.24	
VF-194	5.08	26202	5.21	
VF-195	7.14	31448	5.14	
VF-196	9.68	35740	5.11	
VF-197	5.00	33473	5.03	
VF-198	6.66	45483	4.95	
VF-199	5.74	32454	4.87	
VF-200	4.70	19478	4.70	
VF-201	9.00	32275	4.66	
VF-202	6.38	30254	4.65	
VF-203	4.12	93680	4.62	
VF-204	7.13	32002	4.42	
VF-205	7.49	51535	4.41	
VF-206	4.30	81939	4.36	
VF-207	8.01	34096	4.36	
VF-208	5.57	58693	4.34	
VF-209	9.86	34695	4.33	
VF-210	4.67	65033	4.21	
VF-211	9.36	16491	4.16	
VF-212	9.39	11427	4.11	
VF-213	5.29	54625	4.04	
VF-214	4.94	43667	4.04	
VF-215	9.21	21632	3.95	
VF-216	5.05	153158	3.77	
VF-217	5.85	19316	3.76	
VF-218	5.61	74489	3.74	
VF-219	8.97	17091	3.68	
VF-220	4.16	36450	3.66	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-221	7.27	41723	3.07	
VF-222	6.24	102603	2.82	
VF-223	7.89	57515	2.55	
VF-224	4.67	94138	2.54	
VF-225	7.26	16614	2.48	
VF-226	5.19	12080	2.36	
VF-227	5.04	40716	2.33	0.03038
VF-228	5.08	91613	2.32	0.03734
VF-229	6.21	12903	2.32	
VF-230	5.82	50026	2.27	0.03671
VF-231	4.56	20268	2.24	
VF-232	8.16	34096	2.18	
VF-233	5.03	181267	2.15	
VF-234	5.58	63762	2.10	0.03671
VF-235	4.94	134070	2.04	0.03671
VF-236	6.89	42326	1.99	0.03689
VF-237	4.93	102603	1.97	
VF-238	8.90	15083	1.90	
VF-239	4.26	75782	1.80	
VF-240	6.57	28136	1.77	0.03734
VF-241	4.95	45574	1.76	0.03689
VF-242	4.82	81483	1.72	0.02157
VF-243	4.31	88597	1.71	
VF-244	5.38	26392	1.68	
VF-245	6.18	187641	1.65	
VF-246	5.24	88817	1.65	
VF-247	4.82	30552	1.63	
VF-248	5.48	51880	1.61	0.03689
VF-249	5.10	27209	1.61	
VF-250	6.00	49723	1.59	0.03671
VF-251	6.64	123390	1.58	0.03671
VF-252	5.62	50026	1.58	
VF-253	6.57	20336	1.57	
VF-254	4.46	32643	1.53	0.03671
VF-255	5.05	160613	1.48	
VF-256	6.77	34594	1.44	

VF#	pI	MW (Da)	Fold Decrease	Rank Sum P-Value
VF-257	7.51	27636	1.44	
VF-258	5.19	91103	1.42	0.02157
VF-259	6.28	24374	1.29	
VF-260	7.47	19095	1.21	
VF-261	5.82	41902	1.17	
VF-262	8.17	12814	1.16	
VF-263	5.16	26050	1.15	
VF-264	4.74	30882	1.11	
VF-265	7.96	42551	1.07	

Where p values are given in Table I, the statistical technique used was the Wilcoxon Rank-Sum test as described in method (a) of Section 6.1.13, Statistical Analysis of the Profiles. Where no p value is reported, the method used to select these features was on the basis of a significant fold change or qualitative presence or absence alone as described in methods (b) and (c) of Section 6.1.13 Statistical Analysis of the Profiles.

The second group consists of VFs that are increased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia. These VFs can be described by MW and pI as follows:

Table II. VFs Increased in CSF of Subjects Having Vascular Dementia

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-92	9.80	18109	62.10	
VF-93	9.59	18169	55.37	
VF-94	9.74	56994	27.53	
VF-95	9.47	37090	15.80	
VF-96	4.34	150277	11.95	
VF-97	7.24	11749	40.11	
VF-98	7.48	11668	29.20	
VF-99	4.29	40288	19.05	
VF-100	7.50	55738	18.91	
VF-101	4.32	38629	11.85	
VF-102	5.91	64736	28.05	
VF-103	5.26	10405	17.77	
VF-104	5.82	11533	15.41	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-105	4.45	30662	16.52	
VF-106	3.96	60192	14.22	
VF-107	6.31	17820	13.10	
VF-108	7.88	11369	14.95	
VF-109	6.18	15749	10.74	
VF-110	7.27	14163	16.78	
VF-111	10.21	31005	6.65	
VF-112	6.81	57414	6.16	
VF-113	5.84	39556	7.96	
VF-114	9.81	41481	12.47	
VF-115	5.79	16609	6.00	
VF-116	6.08	10122	8.60	
VF-118	5.79	14996	11.57	
VF-120	6.17	63376	8.02	
VF-121	7.05	11388	7.81	
VF-122	7.44	26066	9.79	
VF-123	6.65	13831	9.31	
VF-124	5.87	45258	6.48	
VF-125	6.80	65526	7.82	
VF-126	8.02	11130	7.59	
VF-127	9.80	18843	9.10	
VF-128	5.61	135816	9.48	
VF-129	5.59	158545	8.51	
VF-130	4.84	118262	6.15	
VF-131	4.39	89827	5.88	
VF-132	6.54	13783	6.07	
VF-134	4.77	99610	8.21	
VF-135	6.45	20882	5.35	0.03689
VF-136	9.58	20268	5.98	0.01996
VF-137	7.26	12594	5.11	0.03689
VF-138	9.22	16179	5.22	0.01219
VF-139	9.22	19032	5.08	0.01945
VF-140	6.11	31600	3.59	0.03689
VF-141	4.40	146682	4.64	0.01996
VF-142	6.35	19414	4.29	0.01219
VF-143	9.58	21021	4.35	0.03689

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-144	7.42	56136	3.85	0.02157
VF-145	8.16	59646	4.57	0.03689
VF-146	6.20	87509	2.77	0.03689
VF-147	6.23	12206	3.82	0.01996
VF-148	5.66	146050	3.92	0.03038
VF-149	6.56	20744	3.18	0.03734
VF-150	7.48	59646	3.79	0.03671
VF-151	7.47	22090	3.88	0.02157
VF-152	6.86	50636	3.08	0.03734
VF-153	6.74	54791	3.16	0.01219
VF-154	7.27	94587	3.16	0.01996
VF-155	6.13	88018	2.71	0.01219
VF-156	5.97	14520	3.04	0.02157
VF-157	9.24	21021	2.92	0.01996
VF-158	9.26	21908	2.88	0.01996
VF-159	9.78	29583	2.61	0.01996
VF-160	6.21	67544	2.11	0.01219
VF-161	6.11	74524	2.11	0.02157
VF-162	6.08	27741	2.10	0.01996
VF-163	6.53	10226	2.69	0.01996
VF-164	8.44	19222	2.41	0.01996
VF-166	5.02	67749	1.71	0.03734
VF-168	5.22	13359	1.55	0.02157
VF-170	4.62	28747	1.35	0.03689
VF-266	7.43	29689	26.49	
VF-267	5.51	76637	21.60	
VF-268	8.10	55548	20.80	
VF-269	7.69	31809	18.25	
VF-270	6.03	89654	17.00	
VF-271	9.08	28954	12.44	
VF-272	6.04	40937	12.28	
VF-273	5.69	16547	10.01	
VF-274	4.12	13577	9.04	
VF-275	4.23	39766	9.01	
VF-276	7.19	20961	8.61	
VF-277	4.24	41761	8.59	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-278	9.81	14171	8.58	
VF-279	4.34	30225	8.28	
VF-280	9.31	11137	8.22	
VF-281	10.06	11587	8.18	
VF-282	5.98	45728	7.95	
VF-283	10.04	14024	7.72	
VF-284	9.98	10735	7.72	
VF-285	5.79	27098	7.67	
VF-286	10.03	17060	7.52	
VF-287	7.83	23298	7.38	
VF-288	9.82	59646	7.09	
VF-289	5.67	12175	6.89	
VF-290	9.81	10923	6.87	
VF-291	9.91	27009	6.54	
VF-292	4.83	56582	6.41	
VF-293	6.05	14637	6.36	
VF-294	6.66	14286	6.35	
VF-295	4.39	30225	6.19	
VF-296	5.14	19356	6.14	
VF-297	5.63	158545	6.06	
VF-298	6.40	13915	6.05	
VF-299	5.49	158545	6.01	
VF-300	6.29	44357	5.98	
VF-301	5.53	10673	5.71	
VF-302	7.65	61670	5.65	
VF-303	9.83	39766	5.36	0.03689
VF-304	5.39	28172	4.94	
VF-305	4.69	53640	4.86	
VF-306	5.85	45882	4.59	0.02157
VF-307	9.19	56533	4.20	
VF-308	6.48	42899	4.01	
VF-309	6.03	13175	3.85	
VF-310	6.61	11467	3.56	
VF-311	6.15	45719	3.35	
VF-312	6.29	93680	3.34	
VF-313	4.53	30225	3.33	

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-314	6.98	59466	3.22	0.03689
VF-315	5.71	45728	3.08	
VF-316	6.08	30920	3.00	0.03734
VF-317	6.09	82463	2.94	
VF-318	6.13	178161	2.79	0.03734
VF-319	7.38	59828	2.74	0.02157
VF-320	5.78	148321	2.72	
VF-321	7.31	11037	2.66	
VF-322	5.20	11309	2.61	
VF-323	5.90	44068	2.51	
VF-324	6.39	44664	2.49	0.04975
VF-325	6.28	178161	2.43	0.01219
VF-326	7.07	39307	2.40	
VF-327	6.05	55588	2.22	
VF-328	6.66	65725	2.15	0.03689
VF-329	9.05	19478	2.07	
VF-330	4.77	18049	2.03	
VF-331	6.28	67135	2.01	0.03671
VF-332	6.22	23973	2.00	
VF-333	7.14	32549	1.93	
VF-334	6.06	11270	1.90	0.03671
VF-335	6.70	38112	1.89	
VF-336	5.75	24567	1.86	
VF-337	6.73	62107	1.83	
VF-338	5.84	11062	1.83	
VF-339	5.75	34874	1.83	
VF-340	6.07	65130	1.76	
VF-341	7.01	40510	1.74	0.03655
VF-342	8.16	24182	1.71	0.03671
VF-343	5.47	51028	1.69	
VF-344	6.66	19935	1.68	0.03689
VF-345	5.50	112518	1.63	
VF-346	5.73	22738	1.62	
VF-347	7.40	114396	1.51	
VF-348	5.10	29902	1.49	
VF-349	6.35	53084	1.48	0.03734

VF#	pI	MW (Da)	Fold Increase	Rank Sum P-Value
VF-350	4.64	119650	1.47	
VF-351	9.07	23405	1.46	
VF-352	6.57	19011	1.42	
VF-353	6.69	39193	1.40	
VF-354	4.36	12420	1.37	
VF-355	6.55	164120	1.34	
VF-356	5.44	53312	1.30	
VF-357	5.62	26300	1.28	
VF-358	4.63	29662	1.28	
VF-359	5.02	80131	1.26	
VF-360	4.57	30225	1.25	
VF-361	4.55	24374	1.24	
VF-362	7.29	20444	1.20	
VF-363	6.17	74110	1.18	
VF-364	4.94	171366	1.12	
VF-365	4.41	24762	1.11	
VF-366	6.38	38294	1.10	
VF-367	5.11	24111	1.08	
VF-368	5.14	13240	1.07	
VF-369	6.84	42786	1.05	
VF-370	4.40	27223	1.04	
VF-371	6.46	34796	1.03	
VF-372	6.76	25490	1.02	

Where p values are given in Table II, the statistical technique used was the Wilcoxon Rank-Sum test as described in method (a) of Section 6.1.13 Statistical Analysis of the Profiles. Where no p value is reported, the method used to select these features was on the basis of fold change or qualitative presence or absence alone as described in methods (b) and (c) of Section 6.1.13 Statistical Analysis of the Profiles.

For any given VF, the signal obtained upon analyzing CSF from subjects having Vascular Dementia relative to the signal obtained upon analyzing CSF from subjects free from Vascular Dementia will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the present invention contemplates that each laboratory will, based

on the present description, establish a reference range for each VF in subjects free from Vascular Dementia according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one control positive CSF sample from a subject known to have Vascular Dementia or at least one control negative CSF sample from a subject known to be free from Vascular Dementia (and more preferably both positive and negative control samples) are included in each batch of test samples analyzed. In one embodiment, the level of expression of a feature is determined relative to a background value, which is defined as the level of signal obtained from a proximal region of the image that (a) is equivalent in area to the particular feature in question; and (b) contains no discernable protein feature. The reference range, depending upon the method of detection used and the conditions under which detection is carried out, can include no feature or isoform present, or non-detectable levels of feature or isoform present. Proteins described by pI and MW provided in Tables I and II can be identified by searching 2D-PAGE databases with those pI and MW values. Examples of such databases are provided on the ExPASy Molecular Biology Server (http://www.expasy.ch) under the "SWISS-2DPAGE" section, and other databases are further referenced on this server. Such databases typically provide interactive 2D gels images for a given set of sample and preparation protocol, and the skilled artisan can obtain information relevant to a given feature by pointing and clicking the appropriate section of the image.

In a preferred embodiment, the signal associated with a VF in the CSF of a subject (*e.g.*, a subject suspected of having or known to have Vascular Dementia) is normalized with reference to one or more ERFs detected in the same 2D gel. As will be apparent to one of ordinary skill in the art, such ERFs may readily be determined by comparing different samples using the Preferred Technology. Suitable ERFs include (but are not limited to) that described in the following table.

Table III. Expression Reference Features

ERF#	pI	MW (Da)
ERF-1	7.34	30993
ERF-2	4.86	60009

As those of skill in the art will readily appreciate, the measured MW and pI of a given feature or protein isoform will vary to some extent depending on the precise protocol used for each step of the 2D electrophoresis and for landmark matching. As used herein, the terms

"MW" and "pI" are defined, respectively, to mean the apparent molecular weight and the apparent isoelectric point of a feature or protein isoform as measured in exact accordance with the Reference Protocol identified in Section 6 below. When the Reference Protocol is followed and when samples are run in duplicate or a higher number of replicates, variation in the measured mean pI of a VF or VPI is typically less than 3% and variation in the measured mean MW of a VF or VPI is typically less than 5%. Where the skilled artisan wishes to deviate from the Reference Protocol, calibration experiments should be performed to compare the MW and pI for each VF or protein isoform as detected (a) by the Reference Protocol and (b) by the deviant protocol.

VFs can be used for detection, prognosis, diagnosis, or monitoring of Vascular Dementia, or for identifying patients most likely to respond to a specific therapeutic treatment, or for drug development. In one embodiment of the invention, CSF from a subject (*e.g.*, a subject suspected of having Vascular Dementia) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265. A decreased abundance of said one or more VFs in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In another embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-

115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372. An increased abundance of said one or more VFs in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In yet another embodiment, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more VFs or any combination of them, whose decreased abundance indicates the presence of Vascular Dementia, *i.e.*, VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265; and (b) one or more VFs or any

combination of them, whose increased abundance indicates the presence of Vascular Dementia *i.e.*, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372.

In yet another embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following VFs: VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194,

VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372 wherein the ratio of the one or more VFs relative to an Expression Reference Feature (ERF) indicates whether Vascular Dementia is present. In a specific embodiment, a decrease in one or more VF/ERF ratios in a test sample relative to the VF/ERF ratios in a control sample or a reference range indicates the presence of Vascular Dementia; VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265 are suitable VFs

for this purpose. In another specific embodiment, an increase in one or more VF/ERF ratios in a test sample relative to the VF/ERF ratios in a control sample or a reference range indicates the presence of Vascular Dementia; VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372 are suitable VFs for this purpose.

In a further embodiment of the invention, CSF from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more VFs, or any combination of them, whose decreased VF/ERF ratio(s) in a test sample relative to the VF/ERF ratio(s) in a control sample indicates the presence of Vascular Dementia, *i.e.*, VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24, VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-

238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265; (b) one or more VFs, or any combination of them, whose increased VF/ERF ratio(s) in a test sample relative to the VF/ERF ratio(s) in a control sample indicates the presence of Vascular Dementia, *i.e.*, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, VF-372.

In a preferred embodiment, CSF from a subject is analyzed for quantitative detection of a plurality of VFs.

5.2 Vascular Dementia-Associated Protein Isoforms (VPIs)

In another aspect of the invention, CSF from a subject, preferably a living subject, is analyzed for quantitative detection of one or more Vascular Dementia-Associated Protein Isoforms (VPIs) for screening or diagnosis of Vascular Dementia, to determine the prognosis of a subject having Vascular Dementia, to monitor the effectiveness of Vascular Dementia therapy, for identifying patients most likely to respond to a particular therapeutic treatment or for drug development. As is well known in the art, a given protein may be expressed as variants (isoforms) that differ in their amino acid composition (*e.g.* as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) or as a result

of differential post- translational modification (*e.g.*, glycosylation, phosphorylation, acylation), or both, so that proteins of identical amino acid sequence can differ in their pI, MW, or both. It follows that differential presence of a protein isoform does not require differential expression of the gene encoding the protein in question. As used herein, the term "Vascular Dementia-Associated Protein Isoform" refers to a protein isoform that is differentially present in CSF from a subject having Vascular Dementia compared with CSF from a subject free from Vascular Dementia. As used herein, the term "isoform" also refers to a protein that exists in only a single form, *i.e.*, it is not expressed as several variants.

Two groups of VPIs have been identified by amino acid sequencing of VFs. VPIs were isolated, subjected to proteolysis, and analyzed by mass spectrometry using the methods and apparatus of the Preferred Technology. One skilled in the art can identify sequence information from proteins analyzed by mass spectrometry and/or tandem mass spectrometry using various spectral interpretation methods and database searching tools. Examples of some of these methods and tools can be found at the Swiss Institute of Bioinformatics web site at http://www.expasy.ch/, and the European Molecular Biology Laboratory web site at www.mann.embl-heidelberg.de/Services/PeptideSearch/. Identification of VPIs was performed primarily using the SEQUEST search program (Eng et al, *J. Am. Soc. Mass Spectrom.* (1994) 5:976-989) with raw, uninterpreted tandem mass spectra of tryptic digest peptides as described in the Examples, *infra*. The first group consists of VPIs that are decreased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia, where the differential presence is significant. The amino acid sequences of tryptic digest peptides of these VPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed in Table IV in addition to the pIs and MWs of these VPIs.

Table IV. VPIs Decreased in CSF of Subjects Having Vascular Dementia

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-4	VPI-2	8.89	11749	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-5	VPI-3	9.16	20681	APEAQVSVQPNFQQDK, AQGFTEDTIVFLPQTDK
VF-12	VPI-6	7.62	70511	IPIEDGSGEVVLSR, TIYTPGSTVLYR, QELSEAEQATR
VF-13	VPI-7	6.18	105482	GCPTEEGCGER, AASGTQNNVLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-14	VPI-8	4.65	102603	HSIFTPETNPR, NLDENYCR, YEFLNGR, CEEDEEFTCR, EQQCVIMAENR
VF-15	VPI-90	5.73	14776	ASSIIDELFQDR
VF-16	VPI-9	4.65	32509	IPTTFENGR
VF-19	VPI-130	8.54	54625	VLLDGVQNPR, TIYTPGSTVLYR
VF-20	VPI-91	6.30	73287	DQYELLCR, FDQFFGEGCAPGSQR
VF-20	VPI-92	6.30	73287	GYTQQLAFR
VF-22	VPI-10	9.04	11790	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-25	VPI-131	9.32	13044	ALDFAVGEYNK
VF-26	VPI-11	9.18	48532	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-27	VPI-93	9.76	13546	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-29	VPI-13	5.26	11388	TMLLQPAGSLGSYSYR
VF-32	VPI-94	6.92	109447	WELCDIPR, CEEDEEFTCR
VF-32	VPI-95	6.92	109447	CFELQEAGPPDCR
VF-35	VPI-133	5.65	13084	AADDTWEPFASGK
VF-36	VPI-14	8.99	61111	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-37	VPI-15	9.80	23795	GFQALGDAADIR
VF-38	VPI-96	8.79	22458	TMLLQPAGSLGSYSYR
VF-41	VPI-17	4.91	38741	KGYTQQLAFR, AGDFLEANYMNLQR, DICEEQVNSLPGSITK, DFDFVPPVVR, GYTQQLAFR
VF-41	VPI-97	4.91	38741	ASSIIDELFQDR, ELDESLQVAER
VF-42	VPI-18	6.15	25232	TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-43	VPI-19	5.67	48092	TVQAVLTVPK, ELLDTVTAPQK, SSFVAPLEK, LAAAVSNFGYDLYR, TSLEDFYLDEER, LSYEGEVTK, DTDTGALLFIGK
VF-43	VPI-98	5.67	48092	LCTVATLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-44	VPI-21	5.65	150534	VQVTSQEYSAR
VF-45	VPI-22	4.53	11037	GCSFLPDPYQK
VF-46	VPI-23	4.86	38741	AGDFLEANYMNLQR, DICEEQVNSLPGSITK, GYTQQLAFR, DFDFVPPVVR, KGYTQQLAFR, RQGALELIK
VF-47	VPI-24	5.49	57515	LPGIVAEGR
VF-47	VPI-99	5.49	57515	GSPAINVAVHVFR
VF-48	VPI-25	4.99	15072	GSPAINVAVHVFR
VF-50	VPI-27	5.48	55124	VLSALQAVQGLLVAQGR, DPTFIPAPIQAK, SLDFTELDVAAEK, ALQDQLVLVAAK
VF-50	VPI-28	5.48	55124	YTFELSR
VF-52	VPI-29	5.37	123390	AFLFQDTPR, LDQCYCER, NNAHGYFK, TCPTCNDFHGLVQK, TYFEGER, HNGQIWVLENDR, YLELESSGHR
VF-53	VPI-31	6.61	72071	TIYTPGSTVLYR, VMQDFFIDLR, QELSEAEQATR, IPIEDGSGEVVLSR
VF-54	VPI-100	4.57	13499	YLGYLEQLLR
VF-55	VPI-32	5.30	49423	LAAAVSNFGYDLYR, ALYYDLISSPDIHGTYK, SSFVAPLEK, ELLDTVTAPQK, DTDTGALLFIGK, LSYEGEVTK, TVQAVLTVPK, TSLEDFYLDEER
VF-55	VPI-33	5.30	49423	TALASGGVLDASGDYR
VF-57	VPI-37	9.18	39998	TLLSVGGWNFGSQR, FPLTNAIK, QHFTTLIK, FSNTDYAVGYMLR, GNQWVGYDDQESVK, EGDGSCFPDALDR, LVMGIPTFGR
VF-58	VPI-38	4.86	135312	AMQHISYLNSR, LICSELNGR, LTVTAYDCGK, YRPAEFHWK, EGLDLQVLEDSGR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				GNLAGLTLR
VF-60	VPI-40	5.66	21021	GSIQVDGEELVSGR, GSIQVDGEDLVTGR, NLVLHSAR, LVSEDPINDGEWHR, YQLGSGEARGSGEAR, FSSGITGCVK, GSVYIGGAPDVATLTGGR
VF-64	VPI-43	5.77	52354	VLSALQAVQGLLVAQGR, DPTFIPAPIQAK, LQAILGVPWK, FMQAVTGWK, ALQDQLVLVAAK, SLDFTELDVAAEK
VF-66	VPI-46	5.01	25963	TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-68	VPI-48	5.66	63184	ADLSGITGAR
VF-68	VPI-49	5.66	63184	FQNALLVR, KVPQVSTPTLVEVSR
VF-68	VPI-50	5.66	63184	GECQAEGVLFFQGDR, YYCFQGNQFLR, NFPSPVDAAFR, DYFMPCPGR, VWVYPPEK, RLWWLDLK
VF-171	VPI-158	8.40	14528	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-172	VPI-159	9.05	18350	APEAQVSVQPNFQQDK, TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-177	VPI-160	4.51	50790	VEQATQAIPMER, VIALINDQR
VF-180	VPI-161	9.12	38200	EGDGSCFPDALDR, TLLSVGGWNFGSQR, FSNTDYAVGYMLR, LVMGIPTFGR, GNQWVGYDDQESVK
VF-181	VPI-162	9.65	65654	SCGLHQLLR, VGDTLNLNLR
VF-188	VPI-163	7.89	29058	TGAQELLR
VF-189	VPI-164	8.54	34332	EELVYELNPLDHR, GLCVATPVQLR
VF-193	VPI-165	9.82	67749	LVGGPMDASVEEEGVR
VF-195	VPI-166	7.14	31448	CSVFYGAPSK, GLQDEDGYR, FACYYPR, VEYGFQVK, ITQVLHFTK
VF-203	VPI-167	4.12	93680	LLDSLPSDTR, FPVFMGR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-204	VPI-168	7.13	32002	CSVFYGAPSK, GLQDEDGYR, VEYGFQVK, ITQVLHFTK
VF-207	VPI-169	8.01	34096	EPFLSCCQFAESLR, SFFPENWLWR, EFHLHLR, FEQLELR, GSFEFPVGDAVSK
VF-209	VPI-170	9.86	34695	FISLGEACK, VFLDCCNYITELR, TGLQEVEVK,
VF-209	VPI-171	9.86	34695	SVNDLYIQK, FPVEMTHNHNFR, TLEAQLTPR, NYNLVESLK
VF-210	VPI-172	4.67	65033	TATSEYQTFFNPR, ELLESYIDGR, SPQELLCGASLISDR
VF-212	VPI-173	9.39	11427	LVGGPMDASVEEEGVR
VF-213	VPI-174	5.29	54625	KFPSGTFEQVSQLVK, KLCMAALK, HLSLLTTLSNR, VCSQYAAYGEK, ELPEHTVK, LCDNLSTK, FEDCCQEK, YTFELSR, THLPEVFLSK,LPEATPTELAK,
VF-213	VPI-175	5.29	54625	DPTFIPAPIQAK, VLSALQAVQGLLVAQGR, SLDFTELDVAAEK
VF-216	VPI-176	5.05	153158	EDYICYAR, IDGDTIIFSNVQER, QPEYAVVQR
VF-216	VPI-177	5.05	153158	QSEDSTFYLGER
VF-216	VPI-178	5.05	153158	WLQGSQELPR
VF-219	VPI-179	8.97	17091	LVGGPMDASVEEEGVR
VF-222	VPI-180	6.24	102603	AASGTQNNVLR, EQTMSECEAGALR
VF-223	VPI-181	7.89	57515	GDYPLEAVR, LFEELVR, GIFPVLCK, DPVQEAWAEDVDLR
VF-226	VPI-182	5.19	12080	GSPAINVAVHVFR
VF-227	VPI-183	5.04	40716	FSSCGGGGGSFGAGGGFGSR
VF-227	VPI-184	5.04	40716	LVPVVNNR
VF-227	VPI-185	5.04	40716	SGNENGEFYLR
VF-228	VPI-186	5.08	91613	GYHLNEEGTR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-230	VPI-187	5.82	50026	YEAAVPDPR, EPGEFALLR, TALASGGVLDASGDYR
VF-232	VPI-188	8.16	34096	EPFLSCCQFAESLR, FEQLELR, GSFEFPVGDAVSK, EELVYELNPLDHR
VF-234	VPI-189	5.58	63762	GECQAEGVLFFQGDR, NFPSPVDAAFR, VWVYPPEK, DYFMPCPGR, RLWWLDLK
VF-234	VPI-190	5.58	63762	YLYEIAR, FQNALLVR, KVPQVSTPTLVEVSR,
VF-235	VPI-191	4.94	134070	GNLAGLTLR, EGLDLQVLEDSGR, QFPTPGIR
VF-237	VPI-192	4.93	102603	TGYYFDGISR, CLAFECPENYR, MCVDVNECQR
VF-239	VPI-193	4.26	75782	FEDGVLDPDYPR
VF-241	VPI-194	4.95	45574	TEQWSTLPPETK, DHAVDLIQK, ADGSYAAWLSR, VLSLAQEQVGGSPEK, AEMADQASAWLTR, QGSFQGGFR
VF-242	VPI-195	4.82	81483	VEQATQAIPMER, VIALINDQR
VF-243	VPI-196	4.31	88597	DFTCVHQALK, TLYSSSPR, LEDMEQALSPSVFK, FQPTLLTLPR
VF-245	VPI-197	6.18	187641	LPPNVVEESAR
VF-246	VPI-198	5.24	88817	TGYYFDGISR, CLAFECPENYR
VF-247	VPI-199	4.82	30552	FSSCGGGGGSFGAGGGF GSR
VF-249	VPI-200	5.10	27209	APEAQVSVQPNFQQDK
VF-250	VPI-201	6.00	49723	YEAAVPDPR, EPGEFALLR, TALASGGVLDASGDYR, VAMHLVCPSR, WVNLPEESLLR
VF-253	VPI-202	6.57	20336	EVDSGNDIYGNPIK, SDGSCAWYR
VF-254	VPI-203	4.46	32643	IPTTFENGR
VF-255	VPI-204	5.05	160613	QYTDSTFR
VF-256	VPI-205	6.77	34594	TSLEDFYLDEER

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-256	VPI-206	6.77	34594	NMQDMVEDYR
VF-258	VPI-207	5.19	91103	GYHLNEEGTR, TGYYFDGISR
VF-260	VPI-208	7.47	19095	LYTLVLTDPDAPSR,
VF-261	VPI-209	5.82	41902	TEDTIFLR
VF-261	VPI-210	5.82	41902	VNEPSILEMSR
VF-262	VPI-211	8.17	12814	LVGGPMDASVEEEGVR
VF-263	VPI-212	5.16	26050	THLAPYSDELR
VF-264	VPI-213	4.74	30882	IPTTFENGR

The second group comprises VPIs that are increased in the CSF of subjects having Vascular Dementia as compared with the CSF of subjects free from Vascular Dementia, where the differential presence is significant. The amino acid sequences of tryptic digest peptides of these VPIs identified by tandem mass spectrometry and database searching are listed in Table V in addition to the pIs and MWs of these VPIs.

Table V. VPIs Increased in CSF of Subjects Having Vascular Dementia

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-92	VPI-56	9.80	18109	RPASPISTIQPK, KLDGICWQVR, SLPVSDSVLSGFEQR, QLYGDTGVLGR
VF-92	VPI-57	9.80	18109	ALDFAVGEYNK, LVGGPMDASVEEEGVR
VF-93	VPI-146	9.59	18169	ISISTSGGSFR
VF-94	VPI-147	9.74	56994	VDFTLSSER, LNMGITDLQGLR, TTNIQGINLLFSSR
VF-95	VPI-108	9.47	37090	PYQYPALTPEQK
VF-97	VPI-58	7.24	11749	VVAGVANALAHK, GTFATLSELHCDK, LLVVYPWTQR
VF-98	VPI-61	7.48	11668	LVGGPMDASVEEEGVR
VF-98	VPI-109	7.48	11668	VHLTPEEK, VVAGVANALAHK, GTFATLSELHCDK, LLVVYPWTQR
VF-99	VPI-62	4.29	40288	WFYIASAFR, TEDTIFLR, EQLGEFYEALDCLR

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
VF-100	VPI-63	7.50	55738	IPIEDGSGEVVLSR, QELSEAEQATR
VF-101	VPI-64	4.32	38629	TEDTIFLR, EQLGEFYEALDCLR
VF-102	VPI-65	5.91	64736	FQNALLVR, VPQVSTPTLVEVSR, LCTVATLR
VF-102	VPI-66	5.91	64736	NFPSPVDAAFR, DYFMPCPGR
VF-104	VPI-272	5.82	11533	AQGFTEDTIVFLPQTDK
VF-105	VPI-110	4.45	30662	NILTSNNIDVK, NPNLPPETVDSLK
VF-106	VPI-111	3.96	60192	EVEELMEDTQHK, DCQPGLCCAFQR, DQDGEILLPR
VF-107	VPI-273	6.31	17820	YTNWIQK
VF-108	VPI-148	7.88	11369	MFLSFPTTK
VF-109	VPI-112	6.18	15749	GLQDEDGYR
VF-112	VPI-149	6.81	57414	VGDTLNLNLR
VF-118	VPI-150	5.79	14996	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-122	VPI-67	7.44	26066	GGPLDGTYR, SADFTNFDPR
VF-122	VPI-68	7.44	26066	SGTASVVCLLNNFYPR, LLIYWASTR,
VF-123	VPI-152	6.65	13831	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-127	VPI-69	9.80	18843	TMLLQPAGSLGSYSYR
VF-127	VPI-70	9.80	18843	LVGGPMDASVEEEGVR
VF-135	VPI-269	6.45	20882	FSNTDYAVGYMLR, LVMGIPTFGR, GNQWVGYDDQESVK
VF-132	VPI-113	6.54	13783	LEEQAQQIR
VF-134	VPI-114	4.77	99610	TGYYFDGISR
VF-136	VPI-71	9.58	20268	TMLLQPAGSLGSYSYR
VF-137	VPI-270	7.26	12594	LYTLVLTDPDAPSR, YVWLVYEQDR
VF-138	VPI-72	9.22	16179	APEAQVSVQPNFQQDK, TMLLQPAGSLGSYSYR
VF-139	VPI-73	9.22	19032	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-140	VPI-271	6.11	31600	SSFVAPLEK,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				TSLEDFYLDEER
VF-141	VPI-75	4.40	146682	FFEECDPNK, AQSIAYHLK
VF-142	VPI-77	6.35	19414	SDGSCAWYR, EVDSGNDIYGNPIK
VF-143	VPI-78	9.58	21021	TMLLQPAGSLGSYSYR
VF-144	VPI-79	7.42	56136	VLLDGVQNPR, IPIEDGSGEVVLSR, TIYTPGSTVLYR, QELSEAEQATR, GLEVTITAR
VF-145	VPI-80	8.16	59646	EYESYSDFER, CEGFVCAQTGR
VF-147	VPI-115	6.23	12206	EGIPPDQQR
VF-147	VPI-116	6.23	12206	FEETTADGR
VF-148	VPI-117	5.66	146050	ALEESNYELEGK
VF-149	VPI-81	6.56	20744	EVDSGNDIYGNPIK, SDGSCAWYR,
VF-150	VPI-118	7.48	59646	LNMGITDLQGLR, GQIVFMNR, EMSGSPASGIPVK
VF-151	VPI-82	7.47	22090	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-152	VPI-153	6.86	50636	FQNALLVR
VF-153	VPI-83	6.74	54791	QELSEAEQATR, VLLDGVQNPR, TIYTPGSTVLYR
VF-153	VPI-119	6.74	54791	ATVVYQGER
VF-154	VPI-84	7.27	94587	QELSEAEQATR, IPIEDGSGEVVLSR, TIYTPGSTVLYR
VF-155	VPI-120	6.13	88018	QDACQGDSGGVFAVR
VF-156	VPI-85	5.97	14520	QPVPGQQMTLK, IWDVVEK
VF-157	VPI-121	9.24	21021	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR, APEAQVSVQPNFQQDK
VF-158	VPI-122	9.26	21908	AQGFTEDTIVFLPQTDK, TMLLQPAGSLGSYSYR
VF-159	VPI-123	9.78	29583	ETAASLLQAGYK, SPQELLCGASLISDR
VF-160	VPI-124	6.21	67544	DGFVQDEGTMFPVGK
VF-161	VPI-268	6.11	74524	HVVPNEVVVQR, AGALNSNDAFVLK,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				TGAQELLR
VF-163	VPI-87	6.53	10226	SCDLALLETYCATPAK, GIVEECCFR
VF-164	VPI-125	8.44	19222	AQGFTEDTIVFLPQTDK
VF-166	VPI-154	5.02	67749	QDIVFDGIAQIR, AFQVWSDVTPLR
VF-166	VPI-155	5.02	67749	DGNTLTYYR, AIEDYINEFSVR
VF-166	VPI-156	5.02	67749	WEDILSDEVNVAR, GVALADFNR
VF-168	VPI-88	5.22	13359	GSPAINVAVHVF AADDTWEPFASGK,
VF-170	VPI-127	4.62	28747	ASSIIDELFQDR
VF-170	VPI-128	4.62	28747	NPNLPPETVDSLK, NILTSNNIDVK, IPTTFENGR
VF-170	VPI-129	4.62	28747	GECQAEGVLFFQGDR
VF-275	VPI-214	4.23	39766	WFYIASAFR, TEDTIFLR, YVGGQEHFAHLLILR, TYMLAFDVNDEK, NWGLSVYADKPETTK, EQLGEFYEALDCLR, SDVVYTDWK
VF-277	VPI-215	4.24	41761	WFYIASAFR, TEDTIFLR, YVGGQEHFAHLLILR, TYMLAFDVNDEK, NWGLSVYADKPETTK, EQLGEFYEALDCLR, SDVVYTDWK
VF-280	VPI-216	9.31	11137	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-281	VPI-217	10.06	11587	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-282	VPI-218	5.98	45728	YICENQDSISSK, DVFLGMFLYEYAR, VPQVSTPTLVEVSR
VF-287	VPI-219	7.83	23298	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK,
VF-290	VPI-220	9.81	10923	LVGGPMDASVEEEGVR
VF-291	VPI-221	9.91	27009	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-292	VPI-222	4.83	56582	ADQVCINLR, SGNENGEFYLR
VF-295	VPI-223	4.39	30225	IPTTFENGR
VF-299	VPI-224	5.49	158545	GAYPLSIEPIGVR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				NNEGTYYSPNYNPQSR, QSEDSTFYLGER
VF-302	VPI-225	7.65	61670	ADSGEGDFLAEGGGVR, GGSTSYGTGSETESPR
VF-303	VPI-226	9.83	39766	LVGGPMDASVEEEGVR, ALDFAVGEYNK
VF-305	VPI-227	4.69	53640	HTLNQIDEVK
VF-306	VPI-228	5.85	45882	YICENQDSISSK, CCAAADPHECYAK, FQNALLVR, VPQVSTPTLVEVSR, AVMDDFAAFVEK
VF-309	VPI-229	6.03	13175	GSPAINVAVHVFR
VF-311	VPI-230	6.15	45719	CCAAADPHECYAK, FQNALLVR, VPQVSTPTLVEVSR
VF-313	VPI-231	4.53	30225	IPTTFENGR
VF-314	VPI-232	6.98	59466	AEFQDALEK, LNMGITDLQGLR, VGDTLNLNLR
VF-316	VPI-233	6.08	30920	TSLEDFYLDEER
VF-319	VPI-234	7.38	59828	HVVPNEVVVQR
VF-324	VPI-235	6.39	44664	IVQLIQDTR, SIPQVSPVR
VF-324	VPI-236	6.39	44664	LVAEFDFR
VF-329	VPI-237	9.05	19478	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-330	VPI-238	4.77	18049	MFGRPWSR
VF-331	VPI-239	6.28	67135	EQTMSECEAGALR,
VF-332	VPI-240	6.22	23973	LVNEVTEFAK, AAFTECCQAADK, SLHTLFGDK
VF-333	VPI-241	7.14	32549	VHYTVCIWR, CSVFYGAPSK, GLQDEDGYR, FACYYPR, VEYGFQVK, ITQVLHFTK
VF-333	VPI-242	7.14	32549	TSLEDFYLDEER
VF-334	VPI-243	6.06	11270	VNHVTLSQPK
VF-335	VPI-244	6.70	38112	VPTVDVSVVDLTVR,
VF-336	VPI-245	5.75	24567	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK,
VF-337	VPI-246	6.73	62107	IPSETLNR
VF-340	VPI-247	6.07	65130	GECQAEGVLFFQGDR,

VF#	VPI#	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides
				NFPSPVDAAFR
VF-341	VPI-248	7.01	40510	LNDLEEALQQAK
VF-342	VPI-249	8.16	24182	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-343	VPI-250	5.47	51028	TSLEDFYLDEER, LAAAVSNFGYDLYR, SSFVAPLEK
VF-344	VPI-251	6.66	19935	ALEESNYELEGK
VF-346	VPI-252	5.73	22738	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-346	VPI-253	5.73	22738	LVNEVTEFAK, YLYEIAR
VF-348	VPI-254	5.10	29902	TGAQELLR
VF-349	VPI-255	6.35	53084	DFYVDENTTVR
VF-351	VPI-256	9.07	23405	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK
VF-352	VPI-257	6.57	19011	EVDSGNDIYGNPIK
VF-353	VPI-258	6.69	39193	INHGILYDEEK, EIMENYNIALR
VF-356	VPI-259	5.44	53312	YTFELSR
VF-356	VPI-260	5.44	53312	DPTFIPAPIQAK, VLSALQAVQGLLVAQGR
VF-359	VPI-261	5.02	80131	HQFLLTGDTQGR, CEGPIPDVTFELLR
VF-360	VPI-262	4.57	30225	IPTTFENGR
VF-361	VPI-263	4.55	24374	TMLLQPAGSLGSYSYR
VF-362	VPI-264	7.29	20444	TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK, APEAQVSVQPNFQQDK
VF-365	VPI-265	4.41	24762	LPYTASSGLMAPR
VF-368	VPI-266	5.14	13240	LVNEVTEFAK
VF-370	VPI-267	4.40	27223	IPTTFENGR

As will be evident to one of skill in the art, based upon the present description, a given VPI can be described according to the data provided for that VPI in Table IV or V. The VPI is a protein comprising a peptide sequence described for that VPI (preferably comprising a plurality of, more preferably all of, the peptide sequences described for that VPI) and has a pI of about the value stated for that VPI (preferably within 10%, more preferably within 5% still more preferably within 1% of the stated value) and has a MW of about the value stated for that VPI (preferably within 10%, more preferably within 5%, still more preferably within 1% of

the stated value). Proteins comprising the peptide sequences provided in Table IV and V can be identified by searching sequence databases with those peptides using search tools known to those skilled in the art. Examples of search algorithm tools that can be used to identify proteins from peptide sequences include:

- BLAST (Basic Local Alignment Search Tool) : BLAST is maintained at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and is based on a statistical theory developed by Samuel Karlin and Steven Altschul (*Proc. Natl Acad. Sci.* USA (1990) 87:2284-2268), later modified as in Karlin and Altschul (*Proc. Natl Acad. Sci.* (1993) 90:5873). BLASTP can be used to search a protein sequence against a protein database. TBLASTN can be used to search a Protein Sequence against a Nucleotide Database, by translating each database Nucleotide sequence in all 6 reading frames.
- FASTA as described in Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-8. See also Pearson *Methods Enzymol.* (1990) 183:63-98 and Pearson *Genomics* (1991) 11(3):635-50.

Examples of available protein sequence databases include:

- The nr protein database maintained at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The nr protein database is compiled of entries from various sources including SwissProt, SwissProt updates, PIR, and PDB. The BLAST resource is available for sequence searching.
- SwissProt and TrEMBL databases developed by the Swiss Bioinformatics Institute (SIB) and the European can be found at http://www.expasy.ch. BLASTP resources are available for sequence searching.
- The PIR-International Protein Sequence Database maintained by the Protein Information Resource (PIR), in collaboration with the Munich Information Center for Protein Sequences (MIPS) and the Japanese International Protein Sequence Database (JIPID). The Protein Identification Resource (PIR) is a division of the National Biomedical Research Foundation (NBRF) which is affiliated with Georgetown University Medical Center and can be found at http://www nbrf.georgetown.edu/pir/searchdb.html. The database can be searched using BLAST and FASTA search algorithm tools.
- The Protein Data Bank, maintained by Brookhaven National Laboratory (Long Island, New York, USA) which can be found at http://www.rcsb.org/pdb/. The FASTA resource is available at this website for sequence searching.

In one embodiment, CSF from a subject is analyzed for quantitative detection of one or more of the following VPIs: VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-130, VPI-131, VPI-133, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, or any combination of them, wherein a decreased abundance of the VPI or VPIs (or any combination of them) in the CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In another embodiment of the invention, CSF from a subject is analyzed for quantitative detection of one or more of the following VPIs: VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, VPI-273, or any combination of them, wherein an increased abundance of the VPI or VPIs (or any combination of them) in CSF from the subject relative to CSF from a subject or subjects free from Vascular Dementia (*e.g.*, a control sample or a previously determined reference range) indicates the presence of Vascular Dementia.

In a further embodiment, CSF from a subject is analyzed for quantitative detection of (a) one or more VPIs, or any combination of them, whose decreased abundance indicates the presence of Vascular Dementia, *i.e.*, VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-130, VPI-131, VPI-133, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213; and (b) one or more VPIs, or any combination of them, whose increased abundance indicates the presence of Vascular Dementia, *i.e.*, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232 VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, VPI-273.

In yet a further embodiment, CSF from a subject is analyzed for quantitative detection of one or more VPIs and one or more previously known biomarkers of Vascular Dementia (*e.g.*, candidate markers such as hypersensitive platelet glutamate receptors (Berk et al, *Int Clin Psychopharmacol* (1999) 14:199-122)). In accordance with this embodiment, the abundance of each VPI and known biomarker relative to a control or reference range indicates whether a subject has Vascular Dementia.

Preferably, the abundance of a VPI is normalized to an Expression Reference Protein Isoform (ERPI). ERPIs can be identified by partial amino acid sequencing of ERFs, which are described above, using the methods and apparatus of the Preferred Technology. The partial amino acid sequences of an ERPI, and the known proteins to which it is homologous is presented in Table VI.

Table VI. Expression Reference Protein Isoforms

ERF#	ERPI #	Amino Acid Sequences of Tryptic Digest Peptides
ERF-1	ERPI-1	GLQDEDGYR
ERF-2	ERPI-2	DPTFIPAPIQAK, ALQDQLVLVAAK

As shown above, the VPIs described herein include previously unknown proteins, as well as isoforms of known proteins where the isoforms were not previously known to be associated with Vascular Dementia. For each VPI, the present invention additionally provides: (a) a preparation comprising the isolated VPI; (b) a preparation comprising one or more fragments of the VPI; and (c) antibodies that bind to said VPI, to said fragments, or both to said VPI and to said fragments. As used herein, a VPI is "isolated" when it is present in a preparation that is substantially free of contaminating proteins, *i.e.*, a preparation in which less than 10% (preferably less than 5%, more preferably less than 1%) of the total protein present is contaminating protein(s). A contaminating protein is a protein or protein isoform having a significantly different pI or MW from those of the isolated VPI, as determined by 2D electrophoresis. As used herein, a "significantly different" pI or MW is one that permits the contaminating protein to be resolved from the VPI on 2D electrophoresis, performed according to the Reference Protocol.

In one embodiment, an isolated protein is provided, said protein comprising a peptide with the amino acid sequence identified in Table IV or V for a VPI, said protein having a pI and MW within 10% (preferably within 5%, more preferably within 1%) of the values identified in Table IV or V for that VPI.

The VPIs of the invention can be qualitatively or quantitatively detected by any method known to those skilled in the art, including but not limited to the Preferred Technology described herein, kinase assays, enzyme assays, binding assays and other functional assays, immunoassays, and western blotting. In one embodiment, the VPIs are separated on a 2-D gel

by virtue of their MWs and pIs and visualized by staining the gel. In one embodiment, the VPIs are stained with a fluorescent dye and imaged with a fluorescence scanner. Sypro Red (Molecular Probes, Inc., Eugene, Oregon) is a suitable dye for this purpose. A preferred fluorescent dye is Pyridinium, 4-[2-[4- (dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt. See U.S. Application No. 09/412,168, filed on October 5, 1999, which is incorporated herein by reference in its entirety.

Alternatively, VPIs can be detected in an immunoassay. In one embodiment, an immunoassay is performed by contacting a sample from a subject to be tested with an anti-VPI antibody under conditions such that immunospecific binding can occur if the VPI is present, and detecting or measuring the amount of any immunospecific binding by the antibody. Anti-VPI antibodies can be produced by the methods and techniques taught herein; examples of such antibodies known in the art are set forth in Table VII. These antibodies shown in Table VII are already known to bind to the protein of which the VPI is itself a family member. Preferably, the anti-VPI antibody preferentially binds to the VPI rather than to other isoforms of the same protein. In a preferred embodiment, the anti-VPI antibody binds to the VPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms of the same protein.

VPIs can be transferred from the gel to a suitable membrane (*e.g.* a PVDF membrane) and subsequently probed in suitable assays that include, without limitation, competitive and non-competitive assay systems using techniques such as western blots and "sandwich" immunoassays using anti-VPI antibodies as described herein, *e.g.*, the antibodies identified in Table VII, or others raised against the VPIs of interest. The immunoblots can be used to identify those anti-VPI antibodies displaying the selectivity required to immuno-specifically differentiate a VPI from other isoforms encoded by the same gene.

Table VII. Known Antibodies That Recognize VPIs or VPI-Related Polypeptides

VPI#	Antibody	Manufacturer	Cat. No.
VPI-2	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-6	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-7	C7 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G34
VPI-8	Monoclonal mouse anti-human plasminogen	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4P11-4D2

VPI#	Antibody	Manufacturer	Cat. No.
VPI-9	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-10	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-11	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-14	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-15	Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP1) (NO X w/TIMP2), Clone: 2A5, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA497
VPI-17	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-23	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-24	Antithrombin III, Clone: BL-ATIII/3, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 9009-1
VPI-25	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-27	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-31	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-43	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-48	Alpha-1-Antichymotrypsin, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 145/2
VPI-49	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-50	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-56	C8 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G35
VPI-57	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL &	AXL- 574

VPI#	Antibody	Manufacturer	Cat. No.
		SCIENTIFIC CORPORATION	
VPI-58	Hemoglobin, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- J16
VPI-61	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-62	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-63	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-64	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-65	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-66	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-67	Kappa Chain, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- 021D
VPI-70	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-77	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-79	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-80	C8 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G35
VPI-81	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-83	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-84	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-87	Insulin Like Growth Factor II (IGF-II), Clone: W2H1, Mab anti-, frozen, IH/ELISA/RIA	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MAS- 976p

VPI#	Antibody	Manufacturer	Cat. No.
VPI-88	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-90	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-91	Monoclonal mouse anti-lactoferrin	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4L2-LF2B8
VPI-92	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-93	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-94	Monoclonal mouse anti-human plasminogen	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK4P11-4D2
VPI-97	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-98	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-99	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-109	Hemoglobin, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- J16
VPI-110	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-112	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-113	Apolipoprotein E, LDL, VLDL, Clone: 3D12, Mab anti-Human, frozen/paraffin	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YM- 5029
VPI-117	ANTI-CYTOKERATIN TYPE 10	RDI RESEARCH DIAGNOSTICS, INC	RDI-CBL196
VPI-118	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-120	C1r Complement, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YSRT- AHC002
VPI-123	Prothrombin, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 448/2
VPI-127	Goat anti-Clusterin (human)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CLUSTRCabG
VPI-128	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457

VPI#	Antibody	Manufacturer	Cat. No.
VPI-129	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-130	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-131	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-133	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-147	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-149	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-153	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-158	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-162	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-163	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-164	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-165	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-166	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-168	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-169	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-170	C3 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-001-02
VPI-171	Heparin Cofactor II, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHCFII
VPI-172	Prothrombin, Rabbit anti-Human	ACCURATE CHEMICAL &	AXL- 448/2

VPI#	Antibody	Manufacturer	Cat. No.
		SCIENTIFIC CORPORATION	
VPI-173	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-175	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-178	Monoclonal mouse anti-human IgA1	RDI RESEARCH DIAGNOSTICS, INC	RDI-TRK1A2-2B5
VPI-179	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-180	C7 Complement, Goat anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BMD- G34
VPI-182	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-183	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-188	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-189	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-190	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-194	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-199	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-202	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-203	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-206	Polylconal Rabbit anti-Human Cytokeratin 1 (Keratin 1)	RDI RESEARCH DIAGNOSTICS, INC	RDI-CYTOK1abr
VPI-209	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-211	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574

VPI#	Antibody	Manufacturer	Cat. No.
VPI-212	Apolipoprotein A (HDL), Sheep anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	ACL- 20075AP
VPI-213	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-214	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-215	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-216	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-217	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-218	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-220	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-221	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-223	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-226	Cystatin C, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	AXL- 574
VPI-228	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-229	Transthyretin, Prealbuminm, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193
VPI-230	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-231	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-232	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-234	Gelsolin, plasma + cytoplasmic, Sh ep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-239	C7 Complement, Goat anti-	ACCURATE CHEMICAL &	BMD- G34

VPI#	Antibody	Manufacturer	Cat. No.
	Human	SCIENTIFIC CORPORATION	
VPI-240	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-241	C4 Complement, Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-032-02
VPI-247	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
VPI-251	ANTI-CYTOKERATIN TYPE 10	RDI RESEARCH DIAGNOSTICS, INC	RDI-CBL196
VPI-253	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-254	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
VPI-255	Monoclonal anti-Prekallikrein Heavy Chain	BIODESIGN INTERNATIONAL	N55199M
VPI-257	Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2) (NO X w/TIMP1), Clone: 3A4, Mab anti-Human, paraffin, IH	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA498
VPI-258	Factor H (Complement), Chicken anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-066-02
VPI-260	AT1 (306)	SANTA CRUZ BIOTECHNOLOGY, INC - RESEARCH ANTIBODIES 98/99	sc-579
VPI-261	Alpha-1-Acid Glycoprotein, Clone: AGP-47, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 6189-1
VPI-262	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-266	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
VPI-267	Apolipoprotein D, Clone: 36C6, Mab anti-Human, paraffin, IH/WB	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA457
VPI-268	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210

*Further information about these antibodies can be obtained from their commercial sources at: ACCURATE CHEMICAL & SCIENTIFIC CORPORATION http://www.accuratechemical.com; BIODESIGN INTERNATIONAL -http://www.biodesign.com/; RDI RESEARCH DIAGNOSTICS, INC - http://www.researchd.com/

In one embodiment, binding of antibody in tissue sections can be used to detect aberrant VPI localization or an aberrant level of one or more VPIs. In a specific embodiment, antibody to a VPI can be used to assay a tissue sample (*e.g.*, a brain biopsy) from a subject for the level of the VPI where an aberrant level of VPI is indicative of Vascular Dementia. As used herein, an "aberrant level" means a level that is increased or decreased compared with the level in a subject free from Vascular Dementia or a reference level. If desired, the comparison can be performed with a matched sample from the same subject, taken from a portion of the body not affected by Vascular Dementia.

Any suitable immunoassay can be used, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement- fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

For example, a VPI can be detected in a fluid sample (*e.g.*, CSF, blood, urine, or tissue homogenate) by means of a two-step sandwich assay. In the first step, a capture reagent (*e.g.*, an anti-VPI antibody) is used to capture the VPI. Examples of such antibodies known in the art are set forth in Table VII. The capture reagent can optionally be immobilized on a solid phase. In the second step, a directly or indirectly labelled detection reagent is used to detect the captured VPI. In one embodiment, the detection reagent is a lectin. Any lectin can be used for this purpose that preferentially binds to the VPI rather than to other isoforms that have the same core protein as the VPI or to other proteins that share the antigenic determinant recognized by the antibody. In a preferred embodiment, the chosen lectin binds to the VPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms that have the same core protein as the VPI or to said other proteins that share the antigenic determinant recognized by the antibody. Based on the present description, a lectin that is suitable for detecting a given VPI can readily be identified by methods well known in the art, for instance upon testing one or more lectins enumerated in Table I on pages 158-159 of Sumar et al, Lectins as Indicators of Disease-Associated Glycoforms, In: Gabius H-J & Gabius S (eds.), 1993, Lectins and Glycobiology, at pp. 158-174 (which is incorporated herein by reference in its entirety). Lectins with the desired oligosaccharide specificity can be identified, for example, by their ability to detect the VPI in a 2D gel, in a replica of a 2D gel following transfer to a suitable solid substrate such as a nitrocellulose membrane, or in a two-step assay following capture by

an antibody. In an alternative embodiment, the detection reagent is an antibody, *e.g.*, an antibody that immunospecifically detects other post-translational modifications, such as an antibody that immunospecifically binds to phosphorylated amino acids. Examples of such antibodies include those that bind to phosphotyrosine (BD Transduction Laboratories, catalog nos.: P11230-050/P11230-150; P11120; P38820; P39020), those that bind to phosphoserine (Zymed Laboratories Inc., South San Francisco, CA, catalog no. 61- 8100) and those that bind to phosphothreonine (Zymed Laboratories Inc., South San Francisco, CA, catalog nos. 71-8200, 13-9200).

If desired, a gene encoding a VPI, a related gene, or related nucleic acid sequences or subsequences, including complementary sequences, can also be used in hybridization assays. A nucleotide encoding a VPI, or subsequences thereof comprising at least 8 nucleotides, preferably at least 12 nucleotides, and most preferably at least 15 nucleotides can be used as a hybridization probe. Hybridization assays can be used for detection, prognosis, diagnosis, or monitoring of conditions, disorders, or disease states, associated with aberrant expression of genes encoding VPIs, or for differential diagnosis of subjects with signs or symptoms suggestive of Vascular Dementia. In particular, such a hybridization assay can be carried out by a method comprising contacting a subject's sample containing nucleic acid with a nucleic acid probe capable of hybridizing to a DNA or RNA that encodes a VPI, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization. Nucleotides can be used for therapy of subjects having Vascular Dementia, as described below.

The methods and compositions for clinical screening, diagnosis and prognosis of Vascular Dementia in a mammalian subject may be diagnostic of Vascular Dementia or indicative of Vascular Dementia.

Diagnostic methods and compositions are based on Vascular Dementia-Associated Features (VFs) and Vascular Dementia-Associated Protein Isoforms (VPIs) which are specifically and particularly associated with Vascular Dementia and are generally not associated with other diseases or conditions. Such diagnostic VFs or VPIs, which are specifically associated with Vascular Dementia, are useful in screening, diagnosis and prognosis as indicators of Vascular Dementia. The administration of therapeutic compositions which are directed against or lead to modulation of diagnostic markers may have therapeutic value particularly in Vascular Dementia.

Indicative methods and compositions are based on Vascular Dementia-Associated Features (VFs) and Vascular Dementia-Associated Protein Isoforms (VPIs) which are

associated with Vascular Dementia but may not be specific only for Vascular Dementia, and may be associated with one or more other diseases or conditions. Such indicative VFs or VPIs, which are associated with Vascular Dementia, but not only with Vascular Dementia, are useful in screening, diagnosis and prognosis as indicators of Vascular Dementia. Indicative methods and compositions are particularly useful in the initial or general screening, diagnosis and prognosis of an individual subject, whereby a first indication of a subset of conditions or diseases, including Vascular Dementia, is thereby provided. Additional assessment utilizing diagnostic or particular Vascular Dementia VFs or VPIs may then be undertaken to provide specific, diagnostic screening, diagnosis and prognosis of the individual subject. The administration of therapeutic compositions which are directed against or lead to modulation of indicative markers may have therapeutic value in Vascular Dementia and other disorders as well, or may be useful therapeutically in more than one disease or condition

Thus, a diagnostic marker changes (increases, decreases or otherwise alters form or character) significantly in only a single disease or condition or in only a small number of conditions, particularly in related conditions. Two such diagnostic markers, VF-37 and VF-50, are provided below in Table VIII.

Table VIII. Example of a diagnostic marker for Vascular Dementia:

Feature #	Isoform #	Fold Change	pI	MW (Da)
VF-50	VPI-27	-2.43	5.48	55124

An indicative marker changes (increases, decreases or otherwise alters form or character) significantly in more than one condition, particularly in Vascular Dementia and one or more other distinct diseases or conditions. One such indicative marker, VF-149, is found to increase in Vascular Dementia and is provided in Table IX. This same marker, identified or characterised by the same pI and MW, is noted as SF-219 as similarly found to be increased in Schizophrenia. The VF-149/SF-219 marker is therefore indicative of Vascular Dementia and/or Schizophrenia.

Table IX: Example of an Indicative Marker for Vascular Dementia:

Feature #	Isoform #	Disease	Fold Change	pI	MW (Da)
VF-149	VPI-81	Vascular Dementia	3.18	6.56	20744
SF-219	SPI-114	Schizophrenia	2.77	6.56	20744

The invention also provides diagnostic kits, comprising an anti-VPI antibody. In addition, such a kit may optionally comprise one or more of the following: (1) instructions for using the anti-VPI antibody for diagnosis, prognosis, therapeutic monitoring or any combination of these applications; (2) a labelled binding partner to the antibody; (3) a solid phase (such as a reagent strip) upon which the anti-VPI antibody is immobilized; and (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof. If no labelled binding partner to the antibody is provided, the anti-VPI antibody itself can be labelled with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

The invention also provides a kit comprising a nucleic acid probe capable of hybridizing to RNA encoding a VPI. In a specific embodiment, a kit comprises in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferably 10-30 nucleotides and still more preferably 10-20 nucleotides) that under appropriate reaction conditions can prime amplification of at least a portion of a nucleic acid encoding a VPI, such as by polymerase chain reaction (see, *e.g.*, Innis et al, 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Qβ replicase, cyclic probe reaction, or other methods known in the art.

Kits are also provided which allow for the detection of a plurality of VPIs or a plurality of nucleic acids each encoding a VPI. A kit can optionally further comprise a predetermined amount of an isolated VPI protein or a nucleic acid encoding a VPI, *e.g.*, for use as a standard or control.

5.3 Statistical Techniques for Identifying VPIs and VPI Clusters

The uni-variate differential analysis tools, such as fold changes, wilcoxon rank sum test and t-test, are useful in identifying individual VFs or VPIs that are diagnostically associated with Vascular Dementia or in identifying individual VPIs that regulate the disease process. In most cases, however, those skilled in the art appreciate that the disease process is associated with a combination of VFs or VPIs (and to be regulated by a combination of VPIs), rather than individual VFs and VPIs in isolation. The strategies for discovering such combinations of VFs and VPIs differ from those for discovering individual VFs and VPIs. In such cases, each individual VF and VPI can be regarded as one variable and the disease can be regarded as a joint, multi-variate effect caused by interaction of these variables.

The following steps can be used to identify markers from data produced by the Preferred Technology.

The first step is to identify a collection of VFs or VPIs that individually show significant association with Vascular Dementia. The association between the identified VFs or VPIs and Vascular Dementia need not be as highly significant as is desirable when an individual VF or VPI is used as a diagnostic. Any of the tests discussed above (fold changes, wilcoxon rank sum test, etc.) can be used at this stage. Once a suitable collection of VFs or VPIs has been identified, a sophisticated multi-variate analysis capable of identifying clusters can then be used to estimate the significant multivariate associations with Vascular Dementia.

Linear Discriminant Analysis (LDA) is one such procedure, which can be used to detect significant association between a cluster of variables (*i.e.*, VFs or VPIs) and Vascular Dementia. In performing LDA, a set of weights is associated with each variable (*i.e.*, VF or VPI) so that the linear combination of weights and the measured values of the variables can identify the disease state by discriminating between subjects having Vascular Dementia and subjects free from Vascular Dementia. Enhancements to the LDA allow stepwise inclusion (or removal) of variables to optimize the discriminant power of the model. The result of the LDA is therefore a cluster of VFs or VPIs which can be used, without limitation, for diagnosis, prognosis, therapy or drug development. Other enhanced variations of LDA, such as Flexible Discriminant Analysis permit the use of non-linear combinations of variables to discriminate a disease state from a normal state. The results of the discriminant analysis can be verified by post-hoc tests and also by repeating the analysis using alternative techniques such as classification trees.

A further category of VFs or VPIs can be identified by qualitative measures by comparing the percentage feature presence of a VF or VPI of one group of samples (*e.g.*, samples from diseased subjects) with the percentage feature presence of a VF or VPI in another group of samples (*e.g.*, samples from control subjects). The "percentage feature presence" of a VF or VPI is the percentage of samples in a group of samples in which the VF or VPI is detectable by the detection method of choice. For example, if a VF is detectable in 95 percent of samples from diseased subjects, the percentage feature presence of that VF in that sample group is 95 percent. If only 5 percent of samples from non-diseased subjects have detectable levels of the same VF, detection of that VF in the sample of a subject would suggest that it is likely that the subject suffers from Vascular Dementia.

5.4 Use in Clinical Studies

The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, *e.g.* to evaluate drugs for therapy of Vascular Dementia. In one

embodiment, candidate molecules are tested for their ability to restore VF or VPI levels in a subject having Vascular Dementia to levels found in subjects free from Vascular Dementia or, in a treated subject (*e.g.* after treatment with antiplatelet agents such as aspirin, Buflomedil (Cucinotta et al. *J Int Med Res* (1992) 20:136-49), neuroprotective agents such as Propentofylline (Rother et al. Ann *N Y Acad Sci* (1996) 777:404-9, Mielke et al. *Alzheimer Dis Assoc Disord* (1998) 12 Suppl 2:S29-35, Rother et al. *Dement Geriatr Cogn Disord* (1998) 9 Suppl 1:36-43), cholinesterase inhibitors such as rivastigmine, galantamine (Kumar et al. *Neurology* (1999) 52 Suppl 2:A395) and other cytoprotective agents currently under clinical evaluation such as the calcium antagonists Nimodipine and Nicadipine, NMDA antagonists such as Selfotel, Dextrorphan, Cerestat, Eliprodil, Lamortigine, GABA agonists, Kappa-selective opiod antagonists, Lubeluzole, Free radicalscavengers, anti-ICAM antibodies and GM-1 ganglioside, Abbokinase®, Activase®,Aggrenox®, Anti-ICAM-1 antibody, Anti-beta-2-integrin antibody, Arvin®, Atacand®, CerAxon®, Cerebyx®, Ceresine®, Cerestat®, Cervene®, Coumadin®, Fiblast®, Fraxiparine®, Freedox®, Innohep®, Kabikinase®, Klerval®, LeukArrest®, Lipitor®, Lovenox®, Neurogard®, Nimotop®, Orgaran®, Persantine®, Plavix®, Prolyse®, Prosynap®, ReoPro®, Selfotel®, Sibelium®, Streptase®, Streptokinase, Sygen®, Ticlid®, Trental®, Viprinex®, Warfarin, Zanaflex®, Zendra®), to preserve VF or VPI levels at or near non-Vascular Dementia values. The levels of one or more VFs or VPIs can be assayed.

In another embodiment, the methods and compositions of the present invention areused to screen candidates for a clinical study to identify individuals having vasculardementia; such individuals can then be excluded from the study or can be placed in aseparate cohort for treatment or analysis. If desired, the candidates can concurrently bescreened to identify individuals with Lewy Body disease and/or senile dementia;procedures for these screens are well known in the art (Harding and Halliday, *Neuropathol. Appl. Neurobiol.* (1998) 24:195-201).

5.5 Purification of VPIs

In particular aspects, the invention provides isolated mammalian VPIs, preferably human VPIs, and fragments thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" as used herein refers to material displaying one or more functional activities associated with a full-length (wild-type) VPI, *e.g.*,

binding to a VPI substrate or VPI binding partner, antigenicity (binding to an anti-VPI antibody), immunogenicity, enzymatic activity and the like.

In specific embodiments, the invention provides fragments of a VPI comprising at least 5 amino acids, at least 10 amino acids, at least 50 amino acids, or at least 75 amino acids. Fragments lacking some or all of the regions of a VPI are also provided, as are proteins (*e.g.*, fusion proteins) comprising such fragments. Nucleic acids encoding the foregoing are provided.

Once a recombinant nucleic acid which encodes the VPI, a portion of the VPI, or a precursor of the VPI is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

The VPIs identified herein can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once a recombinant nucleic acid that encodes the VPI is identified, the entire amino acid sequence of the VPI can be deduced from the nucleotide sequence of the gene coding region contained in the recombinant nucleic acid. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al, 1984, Nature 310:105-111).

In another alternative embodiment, native VPIs can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a preferred embodiment, VPIs are isolated by the Preferred Technology described supra. For preparative-scale runs, a narrow-range "zoom gel" having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated VPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated VPI in a single run. Those of skill in the art will appreciate that a zoom gel can be used in any separation strategy that employs gel isoelectric focusing.

The invention thus provides an isolated VPI, an isolated VPI-related polypeptide, and an isolated derivative or fragment of a VPI or a VPI-related polypeptide; any of the foregoing can be produced by recombinant DNA techniques or by chemical synthetic methods.

5.6 Isolation of DNA Encoding a VPI

Specific embodiments for the cloning of a gene encoding a VPI, are presented below by way of example and not of limitation.

The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding a VPI or a fragment thereof, or a VPI-related polypeptide, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification. The nucleotide sequences of the present invention also permit the identification and cloning of the gene encoding a VPI homolog or VPI ortholog including, for example, by screening cDNA libraries, genomic libraries or expression libraries.

For example, to clone a gene encoding a VPI by PCR techniques, anchored degenerate oligonucleotides (or a set of most likely oligonucleotides) can be designed for all VPI peptide fragments identified as part of the same protein. PCR reactions under a variety of conditions can be performed with relevant cDNA and genomic DNAs (*e.g.*, from brain tissue or from cells of the immune system) from one or more species. Also vectorette reactions can be performed on any available cDNA and genomic DNA using the oligonucleotides (which preferably are nested) as above. Vectorette PCR is a method that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known. Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end. (Arnold C, *PCR Methods Appl.* (1991) 1(1):39-42; Dyer K.D, *Biotechniques*, (1995) 19(4):550-2). Vectorette PCR may pe performed with probes that are, for example, anchored degenerate oligonucleotides (or most likely oligonucleotides) coding for VPI peptide fragments, using as a template a genomic library or cDNA library pools.

Anchored degenerate oligonucleotides (and most likely oligonucleotides) can be designed for all VPI peptide fragments. These oligonucleotides may be labelled and hybridized to filters containing cDNA and genomic DNA libraries. Oligonucleotides to different peptides from the same protein will often identify the same members of the library. The cDNA and genomic DNA libraries may be obtained from any suitable or desired mammalian species, for example from humans.

Nucleotide sequences comprising a nucleotide sequence encoding a VPI or VPI fragment of the present invention are useful for their ability to hybridize selectively with complementary stretches of genes encoding other proteins. Depending on the application, a variety of hybridization conditions may be employed to obtain nucleotide sequences at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identical, or 100% identical, to the sequence of a nucleotide encoding a VPI.

For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, "highly stringent conditions" means hybridization to filter-bound DNA in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65 °C, and washing in 0.1xSSC/0.1% SDS at 68 °C (Ausubel F.M. et al, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent conditions for duplex formation are required. As used herein "moderately stringent conditions" means washing in 0.2xSSC/0.1% SDS at 42 °C (Ausubel et al, 1989, supra). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42 °C for a probe which is 95 to 100% identical to the fragment of a gene encoding a VPI, 37 °C for 90 to 95% identity and 32 °C for 70 to 90% identity.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode parts or the whole of a VPI. Any suitable method for preparing DNA fragments may be used in the present invention. For example, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T4, and yeast artificial chromosome (YAC). (See, *e.g.*, Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II; Ausubel F.M. et al, eds., 1989, Current Protocols in

Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The genomic library may be screened by nucleic acid hybridization to labelled probe (Benton and Davis, *Science* (1977) 196:180; Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA* (1975) 72:3961).

Based on the present description, the genomic libraries may be screened with labelled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the VPI using optimal approaches well known in the art. Any probe used is at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, or at least 100 nucleotides. Preferably a probe is 10 nucleotides or longer, and more preferably 15 nucleotides or longer.

In Tables IV and V above, some VPIs disclosed herein were found to correspond to isoforms of previously identified proteins encoded by genes whose sequences are publicly known. (Sequence analysis and protein identification of VPIs was carried out using the methods described in Section 6.1.14). To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The SWISS-PROT and trEMBL databases (held by the Swiss Institute of Bioinformatics (SIB) and the European Bioinformatics Institute (EBI) which are available at http://www.expasy.ch/) and the GenBank database (held by the National Institute of Health (NIH) which is available at http://www.ncbi.nlm.nih.gov/GenBank/) provide protein sequences for the VPIs listed in Tables IV and V under the following accession numbers and each sequence is incorporated herein by reference:

Table X. Nucleotide sequences encoding VPIs, VPI Related Proteins or ERPIs

VF#	VPI#	Accession Numbers of Identified Sequences
VF-4	VPI-2	P01034
VF-5	VPI-3	P41222
VF-12	VPI-6	P01024
VF-13	VPI-7	P10643
VF-14	VPI-8	P00747
VF-15	VPI-90	P10909
VF-16	VPI-9	P05090
VF-19	VPI-130	P01024
VF-20	VPI-91	P09571

VF#	VPI#	Accession Numbers of Identified Sequences
VF-20	VPI-92	P01024
VF-22	VPI-10	P01034
VF-25	VPI-131	P01034
VF-26	VPI-11	P01034
VF-27	VPI-93	P01034
VF-29	VPI-13	P41222
VF-32	VPI-94	P00747
VF-32	VPI-95	5453874 (gb)
VF-35	VPI-133	P02766
VF-36	VPI-14	P01034
VF-37	VPI-15	P01033
VF-38	VPI-96	P41222
VF-41	VPI-17	P01024
VF-41	VPI-97	P10909
VF-42	VPI-18	P41222
VF-43	VPI-19	P36955
VF-43	VPI-98	P02768
VF-44	VPI-21	Q12860
VF-45	VPI-22	P07602
VF-46	VPI-23	P01024
VF-47	VPI-24	P01008
VF-47	VPI-99	P02766
VF-48	VPI-25	P02766
VF-50	VPI-27	P01019
VF-50	VPI-28	P02774
VF-52	VPI-29	Q99435
VF-53	VPI-31	P01024
VF-54	VPI-100	P02662
VF-55	VPI-32	P36955
VF-55	VPI-33	5802984 (gb)
VF-57	VPI-37	P36222
VF-58	VPI-38	7662374 (gb)
VF-60	VPI-40	P98160
VF-64	VPI-43	P01019
VF-66	VPI-46	P41222
VF-68	VPI-48	P01011

VF#	VPI#	Accession Numbers of Identified Sequences
VF-68	VPI-49	P02768
VF-68	VPI-50	P02790
VF-92	VPI-56	P07360
VF-92	VPI-57	P01034
VF-93	VPI-146	88052
VF-94	VPI-147	P01028
VF-95	VPI-108	P04075
VF-97	VPI-58	P02023
VF-98	VPI-61	P01034
VF-98	VPI-109	P02023
VF-99	VPI-62	P19652
VF-100	VPI-63	P01024
VF-101	VPI-64	P02763
VF-102	VPI-65	P02768
VF-102	VPI-66	P02790
VF-104	VPI-272	P41222
VF-105	VPI-110	P05090
VF-106	VPI-111	7019363 (gb),
VF-107	VPI-273	Q92876
VF-108	VPI-148	P01922
VF-109	VPI-112	P01028
VF-112	VPI-149	P01028
VF-118	VPI-150	P41222
VF-122	VPI-67	P00918
VF-122	VPI-68	229528
VF-123	VPI-152	P41222
VF-127	VPI-69	P41222
VF-127	VPI-70	P01034
VF-132	VPI-113	P02649
VF-134	VPI-114	P23144
VF-135	VPI-269	P36222
VF-136	VPI-71	P41222
VF-137	VPI-270	P30086
VF-138	VPI-72	P41222
VF-139	VPI-73	P41222
VF-140	VPI-271	P36955

VF#	VPI#	Accession Numbers of Identified Sequences
VF-141	VPI-75	809027 (gb)
VF-142	VPI-77	P16035
VF-143	VPI-78	P41222
VF-144	VPI-79	P01024
VF-145	VPI-80	P07358
VF-147	VPI-115	M26880.1
VF-147	VPI-116	P55052
VF-148	VPI-117	P13645
VF-149	VPI-81	P16035
VF-150	VPI-118	P01028
VF-151	VPI-82	P41222
VF-152	VPI-153	P02768
VF-153	VPI-83	P01024
VF-153	VPI-119	P02749
VF-154	VPI-84	P01024
VF-155	VPI-120	P00736
VF-156	VPI-85	P01027
VF-157	VPI-121	P41222
VF-158	VPI-122	P41222
VF-159	VPI-123	P00734
VF-160	VPI-124	899271
VF-161	VPI-268	P06396
VF-163	VPI-87	P01344
VF-164	VPI-125	P41222
VF-166	VPI-154	P08253
VF-166	VPI-155	P02748
VF-166	VPI-156	9368806, 9368807,
VF-168	VPI-88	P02766
VF-170	VPI-127	P10909
VF-170	VPI-128	P05090
VF-170	VPI-129	P02790
VF-171	VPI-158	P01034
VF-172	VPI-159	P41222
VF-177	VPI-160	P51693
VF-180	VPI-161	P36222
VF-181	VPI-162	P01028

VF#	VPI#	Accession Numbers of Identified Sequences
VF-188	VPI-163	P06396
VF-189	VPI-164	P01028
VF-193	VPI-165	P01034
VF-195	VPI-166	P01028
VF-203	VPI-167	P05155
VF-204	VPI-168	P01028
VF-207	VPI-169	P01028
VF-209	VPI-170	P01024
VF-209	VPI-171	P05546
VF-210	VPI-172	P00734
VF-212	VPI-173	P01034
VF-213	VPI-174	181482
VF-213	VPI-175	P01019
VF-216	VPI-176	6651381
VF-216	VPI-177	P00450
VF-216	VPI-178	P01876
VF-219	VPI-179	P01034
VF-222	VPI-180	P10643
VF-223	VPI-181	2117873
VF-226	VPI-182	P02766
VF-227	VPI-183	P04264
VF-227	VPI-184	Q14118
VF-227	VPI-185	Q12805
VF-228	VPI-186	P23142
VF-230	VPI-187	2745741
VF-232	VPI-188	P01028
VF-234	VPI-189	P02790
VF-234	VPI-190	P02768
VF-235	VPI-191	7662374
VF-237	VPI-192	P23142
VF-239	VPI-193	P04004
VF-241	VPI-194	P01028
VF-242	VPI-195	P51693
VF-243	VPI-196	P05155
VF-245	VPI-197	P01023
VF-246	VPI-198	P23142

VF#	VPI#	Accession Numbers of Identified Sequences
VF-247	VPI-199	P04264
VF-249	VPI-200	410564
VF-250	VPI-201	2745741
VF-253	VPI-202	P16035
VF-254	VPI-203	P05090
VF-255	VPI-204	P00450
VF-256	VPI-205	P36955
VF-256	VPI-206	P04264
VF-258	VPI-207	P23142
VF-260	VPI-208	P30086
VF-261	VPI-209	P02763
VF-261	VPI-210	O14791
VF-262	VPI-211	P01034
VF-263	VPI-212	P02647
VF-264	VPI-213	P05090
VF-275	VPI-214	P02763
VF-277	VPI-215	P02763
VF-280	VPI-216	P01034
VF-281	VPI-217	P01034
VF-282	VPI-218	P02768
VF-287	VPI-219	P41222
VF-290	VPI-220	P01034
VF-291	VPI-221	P01034
VF-292	VPI-222	Q12805
VF-295	VPI-223	P05090
VF-299	VPI-224	P00450
VF-302	VPI-225	223918
VF-303	VPI-226	P01034
VF-305	VPI-227	P02765
VF-306	VPI-228	P02768
VF-309	VPI-229	P02766
VF-311	VPI-230	P02768
VF-313	VPI-231	P05090
VF-314	VPI-232	P01028
VF-316	VPI-233	P36955
VF-319	VPI-234	P06396

VF#	VPI#	Accession Numbers of Identified Sequences
VF-324	VPI-235	P15169
VF-324	VPI-236	401767
VF-329	VPI-237	P41222
VF-330	VPI-238	115647.1
VF-331	VPI-239	P10643
VF-332	VPI-240	P02768
VF-333	VPI-241	P01028
VF-333	VPI-242	P36955
VF-334	VPI-243	P01884
VF-335	VPI-244	P08735
VF-336	VPI-245	P41222
VF-337	VPI-246	339568
VF-340	VPI-247	P02790
VF-341	VPI-248	P35908
VF-342	VPI-249	P41222
VF-343	VPI-250	P36955
VF-344	VPI-251	P13645
VF-346	VPI-252	P41222
VF-346	VPI-253	P02768
VF-348	VPI-254	P06396
VF-349	VPI-255	P29622
VF-351	VPI-256	P41222
VF-352	VPI-257	P16035
VF-353	VPI-258	Q03591
VF-356	VPI-259	P02774
VF-356	VPI-260	P01019
VF-359	VPI-261	P04217
VF-360	VPI-262	P05090
VF-361	VPI-263	P41222
VF-362	VPI-264	P41222
VF-365	VPI-265	AK026519.1
VF-368	VPI-266	P02768
VF-370	VPI-267	P05090
ERF-1	ERPI-1	P01028
ERF-2	ERPI-2	P01019

For any VPI, degenerate probes, or probes taken from the sequences described above by accession number may be used for screening. In the case of degenerate probes, they can be constructed from the partial amino sequence information obtained from tandem mass spectra of tryptic digest peptides of the VPI. To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. When a library is screened, clones with insert DNA encoding the VPI or a fragment thereof will hybridize to one or more members of the corresponding set of degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries is carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50 °C and washed using the washing conditions described supra for highly stringent or moderately stringent hybridization.

In yet another aspect of the invention, clones containing nucleotide sequences encoding the entire VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide any of the foregoing may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed VPI or VPI-related polypeptides. In one embodiment, the various anti-VPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988 Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Colonies or plaques from the library are brought into contact with the antibodies to identify those clones that bind antibody.

In an embodiment, colonies or plaques containing DNA that encodes a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide can be detected using DYNA Beads according to Olsvick et al, 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-VPI antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads are then contacted with colonies or plaques expressing recombinant polypeptides. Colonies or plaques expressing a VPI or VPI-related polypeptide are identified as any of those that bind the beads.

Alternatively, the anti-VPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite® resin. This material is then used to adsorb to bacterial colonies expressing the VPI protein or VPI-related polypeptide as described herein.

In another aspect, PCR amplification may be used to isolate from genomic DNA a substantially pure DNA (*i.e.*, a DNA substantially free of contaminating nucleic acids) encoding the entire VPI or a part thereof. Preferably such a DNA is at least 95% pure, more preferably at least 99% pure. Oligonucleotide sequences, degenerate or otherwise, that correspond to peptide sequences of VPIs disclosed herein can be used as primers.

PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp® or AmpliTaq DNA polymerase). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding a VPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

The gene encoding a VPI can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified DNA encoding a VPI of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies that specifically recognize a VPI. A radiolabelled cDNA encoding a VPI can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the DNA fragments encoding a VPI from among other genomic DNA fragments.

Alternatives to isolating genomic DNA encoding a VPI include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA that encodes the VPI. For example, RNA for cDNA cloning of the gene encoding a VPI can be isolated from cells that express the VPI. Those skilled in the art will understand

from the present description that other methods may be used and are within the scope of the invention.

Any suitable eukaryotic cell can serve as the nucleic acid source for the molecular cloning of the gene encoding a VPI. The nucleic acid sequences encoding the VPI can be isolated from vertebrate, mammalian, primate, human, porcine, bovine, feline, avian, equine, canine or murine sources. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, *e.g.*, Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences.

The identified and isolated gene or cDNA can then be inserted into any suitable cloning vector. A large number of vector-host systems known in the art may be used. As those skilled in the art will appreciate, the only limitation is that the vector system chosen be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene) or modified viruses such as adenoviruses, adeno-associated viruses or retroviruses. The insertion into a cloning vector can be accomplished, for example, by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used t fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and the gene encoding a VPI may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated gene encoding the VPI, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from

the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The nucleotide sequences of the present invention include nucleotide sequences encoding amino acid sequences with substantially the same amino acid sequences as native VPIs, nucleotide sequences encoding amino acid sequences with functionally equivalent amino acids, nucleotide sequences encoding VPIs, a fragments of VPIs, VPI- related polypeptides, or fragments of VPI-related polypeptides.

In a specific embodiment, an isolated nucleic acid molecule encoding a VPI- related polypeptide can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of a VPI such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Standard techniques known to those of skill in the art can be used to introduce mutations, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

5.7 Expression of DNA Encoding VPIs

The nucleotide sequence coding for a VPI, a VPI analog, a VPI-related peptide, or a fragment or other derivative of any of the foregoing, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and

translational signals can also be supplied by the native gene encoding the VPI or its flanking regions, or the native gene encoding the VPI- related polypeptide or its flanking regions. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, a nucleotide sequence encoding a human gene (or a nucleotide sequence encoding a functionally active portion of a human VPI) is expressed. In yet another embodiment, a fragment of a VPI comprising a domain of the VPI is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a VPI or fragment thereof may be regulated by a second nucleic acid sequence so that the VPI or fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a VPI may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding a VPI or a VPI- related polypeptide include, but are not limited to, the SV40 early promoter regic (Bernoist and Chambon *Nature* (1981) 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al, *Cell* (1980) 22:787-797), the herpes thymidine kinase promoter (Wagner et al, *Proc. Natl. Acad. Sci. USA* (1981) 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, *Nature* (1982) 296:39-42), the tetracycline (Tet) promoter (Gossen et al, *Proc. Nat. Acad. Sci. USA* (1995) 89:5547-5551); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al, *Proc. Natl. Acad. Sci. USA* (1978) 75:3727-3731), or the tac promoter (DeBoer, et al, *Proc. Natl. Acad. Sci. USA* (1983) 80:21-25; see also "Useful proteins from recombinant bacteria" in *Scientific American* (1980) 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al, *Nature* (1984) 310(5973):115-20) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al, *Nucl. Acids Res.* (1981) 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-

Estrella et al, *Nature* (1984) 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al, *Cell* (1984) 38:639-646; Ornitz et al, *Cold Spring Harbor Symp. Quant. Biol.* (1986) 50:399-409; MacDonald, *Hepatology* (1987) 7:425- 515); insulin gene control region which is active in pancreatic beta cells (Hanahan, *Nature* (1985) 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al, *Cell* (1984) 38:647-658; Adames et al, 1985, Nature 318:533-538; Alexander et al, *Mol. Cell. Biol.* (1987) 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al, *Cell* (1986) 45:485-495), albumin gene control region which is active in liver (Pinkert et al, *Genes and Devel.* (1987) 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al, *Mol. Cell. Biol.* (1985) 5:1639-1648; Hammer et al, *Science* (1987) 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, *Genes and Devel.* (1987) 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al, *Nature* (1985) 315:338-340; Kollias et al, *Cell* (1986) 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al, *Cell* (1987) 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* (1985) 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al, *Gen. Virol.* (1999) 80:571-83); brain-derived neurotrophic factor (BDNF) gene control region which is active in neuronal cells (Tabuchi et al, *Biochem. Biophysic. Res. Com.* (1998) 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al, *Braz J Med Biol Res* (1999) 32(5):619-631; Morelli et al, *Gen. Virol.* (1999) 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al, *Science* (1986) 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a VPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a VPI or a VPI-related polypeptide coding sequence into the EcoRI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, *Gene*

(1988) 7:31-40). This allows for the expression of the VPI product or VPI-related polypeptide from the subclone in the correct reading frame.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the VPI coding sequence or VPI-related polypeptide coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (*e.g.*, see Logan & Shenk, *Proc. Natl. Acad. Sci. USA* (1984) 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al, *Methods in Enzymol.* (1987) 153:51-544).

Expression vectors containing inserts of a gene encoding a VPI or a VPI- related polypeptide can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a VPI inserted in an expression vector ca be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding a VPI. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a gene encoding a VPI in the vector. For example, if the gene encoding the VPI is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the VPI insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (*i.e.*, VPI) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the VPI in *in vitro* assay systems, *e.g.*, binding with anti-VPI antibody.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered VPI or VPI-related polypeptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, Hela, COS, MDCK, 293, 3T3, WI38, and in particular, neuronal cell lines such as, for example, SK-N-AS, SK-N-FI, SK-N-DZ human neuroblastomas (Sugimoto et al, *J. Natl. Cancer Inst.* (1984) 73: 51-57), SK-N-SH human neuroblastoma (Biochim. Biophys. Acta, 1982, 704: 450-460), Daoy human cerebellar medulloblastoma (He et al, *Cancer Res.* (1992) 52: 1144-1148) DBTRG-05MG glioblastoma cells (Kruse et al, *In vitro Cell. Dev. Biol.* (1992) 28A: 609-614), IMR-32 human neuroblastoma (*Cancer Res.*, (1970) 30:2110-2118), 1321N1 human astrocytoma (*Proc. Natl Acad. Sci. USA* (1977) 74:4816), MOG-G-CCM human astrocytoma (*Br. J. Cancer*, (1984) 49:269), U87MG human glioblastoma-astrocytoma (*Acta Pathol. Microbiol. Scand.*, (1968) 74: 465-486), A172 human glioblastoma (Olopade et al, *Cancer Res.* (1992) 52:2523- 2529), C6 rat glioma cells (Benda et al, *Science* (1968) 161:370-371), Neuro-2a mouse neuroblastoma (*Proc. Natl. Acad. Sci. USA*, (1970) 65: 129-136), NB41A3 mouse neuroblastoma (*Proc. Natl. Acad. Sci. USA*, (1962) 48:1184-1190), SCP sheep choroid plexus (Bolin et al, *J. Virol. Methods* (1994) 48: 211-221), G355-5, PG-4 Cat normal astrocyte (Haapala et al, *J. Virol.* (1985) 53:827-833), Mpf ferret brain (Trowbridge et al, *In vitro* (1982) 18:952-960), and normal cell lines such as, for example, CTX TNA2 rat normal cortex brain (Radany et al, *Proc. Natl. Acad. Sci. USA* (1992) 89:6467-6471) such as, for example, CRL7030 and Hs578Bst. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate

expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al, *Cell* (1977) 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* (1962) 48:2026), and adenine phosphoribosyltransferase (Lowy, et al, *Cell* (1980) 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al, *Proc. Natl. Acad. Sci. USA* (1980) 77:3567; O'Hare, et al, *Proc. Natl. Acad. Sci. USA* (1981) 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* (1981) 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre- Garapin, et al, *J. Mol. Biol.* (1981) 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al, *Gene* (1984) 30:147) genes.

In other specific embodiments, the VPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence). For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system. An increase in the half-life *in vivo* and facilitated purification has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, *e.g.*, EP 394,827; Traunecker et al, *Nature*, (1988) 331:84-86 . Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (*e.g.*, insulin) conjugated to an FcRn

binding partner such as IgG or Fc fragments (see, *e.g.*, PCT publications WO 96/22024 and WO 99/04813).

Nucleic acids encoding a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide can be fused to an epitope tag (*e.g.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al, allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al, *Proc. Natl. Acad. Sci. USA* (1991) 88:8972-897).

Fusion proteins can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, a fusion protein may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.8 Domain Structure of VPIs

Domains of some VPIs are known in the art and have been described in the scientific literature. Moreover, domains of a VPI can be identified using techniques known to those of skill in the art. For example, one or more domains of a VPI can be identified by using one or more of the following programs: ProDom, TMpred, and SAPS. ProDom compares the amino acid sequence of a polypeptide to a database of compiled domains (see, *e.g.*, http://www.toulouse.inra.fr/prodom.html; Corpet F., Gouzy J. & Kahn D., *Nucleic Acids Res.*, (1999) 27:263-267). TMpred predicts membrane-spanning regions of a polypeptide and their orientation. This program uses an algorithm that is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins (see, *e.g.*, http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann & Stoffel. (1993) "TMbase - A database of membrane spanning proteins segments." Biol. Chem. Hoppe-Seyler 347,166). The SAPS program analyzes polypeptides for statistically significant features like charge-clusters, repeats, hydrophobic regions, compositional domains (see, *e.g.*, Brendel et al, *Proc. Natl. Acad. Sci. USA* (1992) 89: 2002-2006). Thus, based on the present description, the skilled artisan can identify domains of a VPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of a VPI fragment that retains the enzymatic or binding activity of the VPI.

Based on the present description, the skilled artisan can identify domains of a VPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of VPI fragments that retain the enzymatic or binding activity of the VPI.

In one embodiment, a VPI has an amino acid sequence sufficiently similar to an identified domain of a known polypeptide. As used herein, the term "sufficiently similar" refers to a first amino acid or nucleotide sequence which contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have or encode a common structural domain or common functional activity or both.

A VPI domain can be assessed for its function using techniques well known to those of skill in the art. For example, a domain can be assessed for its kinase activity or for its ability to bind to DNA using techniques known to the skilled artisan. Kinase activity can be assessed, for example, by measuring the ability of a polypeptide to phosphorylate a substrate. DNA binding activity can be assessed, for example, by measuring the ability of a polypeptide to bind to a DNA binding element in a electromobility shift assay. In a preferred embodiment, the function of a domain of a VPI is determined using an assay described in one or more of the references identified in Table XI, *infra*.

5.9 Production of Antibodies to VPIs

According to the invention a VPI, VPI analog, VPI-related protein or a fragment or derivative of any of the foregoing may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such immunogens can be isolated by any convenient means, including the methods described above. Antibodies of the invention include, but are not limited to polyclonal, monoclonal, bispecific, humanized or chimeric antibodies, single chain antibodies, Fab fragments and F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any class (*e.g.*, IgG, IgE, IgM, IgD and IgA) or subclass of immunoglobulin molecule.

In one embodiment, antibodies that recognize gene products of genes encoding VPIs are publicly available. For example, antibodies that recognize these VPIs and/or their isoforms include antibodies recognizing: VPI-2, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-14, VPI-15, VPI-17, VPI-23, VPI-24, VPI-25, VPI-27, VPI-31, VPI-43, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-70, VPI-77, VPI-79, VPI-80, VPI-81, VPI-83, VPI-84, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-97, VPI-98, VPI-99, VPI-109, VPI-110, VPI-112, VPI-113, VPI-117, VPI-118, VPI-120, VPI-123, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-147, VPI-149, VPI-153, VPI-158, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-175, VPI-178, VPI-179, VPI-180, VPI-182, VPI-183, VPI-188, VPI-189, VPI-190, VPI-194, VPI-199, VPI-202, VPI-203, VPI-206, VPI-209, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-220, VPI-221, VPI-223, VPI-226, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-234, VPI-239, VPI-240, VPI-241, VPI-247, VPI-251, VPI-253, VPI-254, VPI-255, VPI-257, VPI-258, VPI-260, VPI-261, VPI-262, VPI-266, VPI-267, VPI-268, which antibodies can be purchased from commercial sources as shown in Table VII above. In another embodiment, methods known to those skilled in the art are used to produce antibodies that recognize a VPI, a VPI analog, a VPI-related polypeptide, or a derivative or fragment of any of the foregoing.

In one embodiment of the invention, antibodies to a specific domain of a VPI are produced. In a specific embodiment, hydrophilic fragments of a VPI are used as immunogens for antibody production.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a VPI, one may assay generated hybridomas for a product which binds to a VPI fragment containing such domain. For selection of an antibody that specifically binds a first VPI homolog but which does not specifically bind to (or binds less avidly to) a second VPI homolog, one can select on the basis of positive binding to the first VPI homolog and a lack of binding to (or reduced binding to) the second VPI homolog. Similarly, for selection of an antibody that specifically binds a VPI but which does not specifically bind to (or binds less avidly to) a different isoform of the same protein (such as a different glycoform having the same core peptide as the VPI), one can select on the basis of positive binding to the VPI and a lack of binding to (or reduced binding to) the different isoform (*e.g.*, a different glycoform). Thus, the present invention

provides an antibody (preferably a monoclonal antibody) that binds with greater affinity (preferably at least 2-fold, more preferably at least 5-fold still more preferably at least 10-fold greater affinity) to a VPI than to a different isoform or isoforms (*e.g.*, glycoforms) of the VPI.

Polyclonal antibodies that may be used in the methods of the invention are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Unfractionated immune serum can also be used. Various procedures known in the art may be used for the production of polyclonal antibodies to a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a VPI or a VPI- related polypeptide can be obtained. For example, for the production of polyclonal or monoclonal antibodies, various host animals can be immunized by injection with the native or a synthetic (*e.g.*, recombinant) version of a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide, including but not limited to rabbits, mice, rats, etc. The Preferred Technology described herein provides isolated VPIs suitable for such immunization. If the VPI is purified by gel electrophoresis, the VPI can be used for immunization with or without prior extraction from the polyacrylamide gel. Various adjuvants may be used to enhance the immunological response, depending on the host species, including, but not limited to, complete or incomplete Freund's adjuvant, a mineral gel such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyol, a polyanion, a peptide, an oil emulsion, keyhole limpet hemocyanin, dinitrophenol, and an adjuvant such as BCG (bacille Calmette-Guerin) or corynebacterium parvum. Additional adjuvants are also well known in the art.

For preparation of monoclonal antibodies (mAbs) directed toward a VPI, a fragment of a VPI, a VPI-related polypeptide, or a fragment of a VPI-related polypeptide, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al, *Immunology Today* (1983) 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAbs of the invention may be cultivated *in vitro* or *in vivo*. In an

additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing known technology (PCT/US90/02545, incorporated herein by reference).

The monoclonal antibodies include but are not limited to human monoclonal antibodies and chimeric monoclonal antibodies (*e.g.*, human-mouse chimeras). A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al, U.S. Patent No. 4,816,567; and Boss et al, U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non- human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.)

Chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al, 1988, Science 240:1041-1043; Liu et al, *Proc. Natl. Acad. Sci. USA* (1987) 84:3439-3443; Liu et al, *J. Immunol.* (1987) 139:3521-3526; Sun et al, *Proc. Natl. Acad. Sci. USA* (1987) 84:214-218; Nishimura et al, *Canc. Res.* (1987) 47:999-1005; Wood et al, *Nature* (1985) 314:446-449; and Shaw et al, *J. Natl. Cancer Inst.* (1988) 80:1553-1559; Morrison, *Science* (1985) 229:1202-1207; Oi et al, *Bio/Techniques* (1986) 4:214; U.S. Patent 5,225,539; Jones et al, *Nature* (1986) 321:552-525; Verhoeyan et al, *Science* (1988) 239:1534; and Beidler et al, *J. Immunol.* (1988) 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Such antibodies can be produced using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a VPI of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human

antibodies, see Lonberg and Huszar (*Int. Rev. Immunol.* (1995) 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, *e.g.*, U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al, *Bio/technology* (1994) 12:899-903).

The antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles that carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labelled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al, *J. Immunol. Methods* (1995) 182:41-50 ; Ames et al, *J. Immunol. Methods* (1995) 184:177-186 ; Kettleborough et al, *Eur. J. Immunol.* (1994) 24:952-958 ; Persic et al, *Gene* (1997) 187 9-18 ; Burton et al, *Advances in Immunology* (1994) 57:191-280 ; PCT Application No. PCT/GB91/01134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired

host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al, BioTechniques 12(6):864-869 (1992); and Sawai et al, (1995) AJRI 34:26-34 ; and Better et al, *Science* (1988) 240:1041-1043 (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al, Methods in Enzymology 203:46-88 (1991); Shu et al, *Proc. Natl. Sci Acad. USA* (1993) 90:7995-7999; and Skerra et al, *Science* (1988) 240:1038-1040 .

The invention further provides for the use of bispecific antibodies, which can be made by methods known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al, *Nature* (1983) 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al, *EMBO J.* (1991) 10:3655-3659 .

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3,1994. For further details for generating bispecific antibodies see, for example, Suresh et al, *Methods in Enzymology* (1986) 121:210.

The invention provides functionally active fragments, derivatives or analogs of the anti-VPI immunoglobulin molecules. Functionally active means that the fragment, derivative or analog is able to elicit anti-anti-idiotype antibodies (*i.e.*, tertiary antibodies) that recognize the same antigen that is recognized by the antibody from which the fragment, derivative or analog is derived. Specifically, in a preferred embodiment the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art.

The present invention provides antibody fragments such as, but not limited to, F(ab')2 fragments and Fab fragments. Antibody fragments which recognize specific epitopes may be generated by known techniques. F(ab')2 fragments consist of the variable region, the light chain constant region and the CH1 domain of the heavy chain and are generated by pepsin digestion of the antibody molecule. Fab fragments are generated by reducing the disulfide bridges of the F(ab')2 fragments. The invention also provides heavy chain and light chain dimers of the antibodies of the invention, or any minimal fragment thereof such as Fvs or single chain antibodies (SCAs) (*e.g.*, as described in U.S. Patent 4,946,778; Bird, *Science* (1988) 242:423-42; Huston et al, *Proc. Natl. Acad. Sci. USA* (1988) 85:5879-5883; and Ward et al, *Nature* (1989) 334:544-54), or any other molecule with the same specificity as the antibody of the invention. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in E. coli may be used (Skerra et al, *Science* (1988) 242:1038-1041).

In other embodiments, the invention provides fusion proteins of the immunoglobulins of the invention (or functionally active fragments thereof), for example in which the immunoglobulin is fused via a covalent bond (*e.g.*, a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably at least 10, 20 or 50 amino acid portion of the protein) that is not the immunoglobulin. Preferably the immunoglobulin, or fragment thereof, is covalently linked to the other protein at the N-terminus of the constant domain. As stated above, such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system.

The immunoglobulins of the invention include analogs and derivatives that are either modified, i.e, by the covalent attachment of any type of molecule as long as such covalent attachment that does not impair immunospecific binding. For example, but not by way of limitation, the derivatives and analogs of the immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the VPIs of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

5.10 Expression Of Antibodies

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression techniques.

Recombinant expression of antibodies, or fragments, derivatives or analogs thereof, requires construction of a nucleic acid that encodes the antibody. If the nucleotide sequence of the antibody is known, a nucleic acid encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al, *BioTechniques* (1994) 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, the nucleic acid encoding the antibody may be obtained by cloning the antibody. If a clone containing the nucleic acid encoding the particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the antibody may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the antibody) by PCR amplification using synthetic primers hybridizable to the 3′ and 5′ ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence.

If an antibody molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an antibody), antibodies specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies. Alternatively, a clone encoding at least the Fab portion of the antibody may be obtained by screening Fab expression libraries (*e.g.*, as described in Huse et al, *Science* (1989) 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, *e.g.*, Clackson et al, *Nature* (1991) 352:624; Hane et al, *Proc. Natl. Acad. Sci. USA* (1997) 94:4937).

Once a nucleic acid encoding at least the variable domain of the antibody molecule is obtained, it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the antibody molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available. Then, the nucleic acid encoding the antibod can be used to introduce the nucleotide substitution(s) or deletion(s) necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydyl group. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a nucleotide sequence, for example, but not limited to, chemical mutagenesis, *in vitro* site directed mutagenesis (Hutchinson et al, *J. Biol. Chem.* (1978) 253:6551), PCT based methods, etc.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al, *Proc. Natl. Acad. Sci.* (1984) 81:851-855; Neuberger et al, *Nature* (1984) 312:604-608; Takeda et al, *Nature* (1985) 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a

chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human antibody constant region, *e.g.*, humanized antibodies.

Once a nucleic acid encoding an antibody molecule of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protein of the invention by expressing nucleic acid containing the antibody molecule sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing an antibody molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al, (1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Ausubel et al, (eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention.

The host cells used to express a recombinant antibody of the invention may be either bacterial cells such as Escherichia coli, or, preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al, *Gene* (1986) 45:101; Cockett et al, *Bio/Technology* (1990) 8:2).

A variety of host-expression vector systems may be utilized to express an antibody molecule of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g.*, E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell

systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions comprising an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al, *EMBO J.* (1983) 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.* (1985) 13:3101-3109; Van Heeke & Schuster, *J. Biol. Chem.* (1989) 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned individually into non- essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). In mammalian host cells, a number of viral-based expression systems (*e.g.*, an adenovirus expression system) may be utilized.

As discussed above, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein.

For long-term, high-yield production of recombinant antibodies, stable expression is preferred. For example, cells lines that stably express an antibody of interest can be produced by transfecting the cells with an expression vector comprising the nucleotide sequence of the antibody and the nucleotide sequence of a selectable (*e.g.*, neomycin or hygromycin), and selecting for expression of the selectable marker. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

The expression levels of the antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al, 1983, Mol. Cell. Biol. 3:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, *Nature* (1986) 322:52; Kohler, *Proc. Natl. Acad. Sci. USA* (1980) 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once the antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an antibody molecule, for example, by chromatography (*e.g.*, ion exchange chromatography, affinity chromatography such as with protein A or specific antigen, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al, allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al, *Proc. Natl. Acad. Sci. USA* (1991) 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six

histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni2+ nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

5.11 Conjugated Antibodies

In a preferred embodiment, anti-VPI antibodies or fragments thereof are conjugated to a diagnostic or therapeutic moiety. The antibodies can be used for diagnosis or to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include 125I, 131I, 111In and 99Tc.

Anti-VPI antibodies or fragments thereof can be conjugated to a therapeutic agent o drug moiety to modify a given biological response. The therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.*, angiostatin or endostatin; or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), nerve growth factor (NGF) or other growth factor.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon et al, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer

Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al, (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al, (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al, (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabelled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al, (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

An antibody with or without a therapeutic moiety conjugated to it can be used as a therapeutic that is administered alone or in combination with cytotoxic factor(s) and/or cytokine(s).

5.12 Diagnosis of Vascular Dementia

In accordance with the present invention, test samples of cerebrospinal fluid (CSF), serum, plasma or urine obtained from a subject suspected of having or known to have Vascular Dementia can be used for diagnosis or monitoring. In one embodiment, a decreased abundance of one or more VFs or VPIs (or any combination of them) in a test sample relative to a control sample (from a subject or subjects free from Vascular Dementia) or a previously determined reference range indicates the presence of Vascular Dementia; VFs and VPIs suitable for this purpose are identified in Tables I and IV, respectively, as described in detail above. In another embodiment of the invention, an increased abundance of one or more VFs or VPIs (or any combination of them) in a test sample compared to a control sample or a previously determined reference range indicates the presence of Vascular Dementia; VFs and VPIs suitable for this purpose are identified in Tables II and V, respectively, as described in detail above. In another embodiment, the relative abundance of one or more VFs or VPIs (or any combination of them) in a test sample compared to a control sample or a previously determined reference range indicates a subtype of Vascular Dementia (*e.g.*, familial or sporadic Vascular Dementia). In yet another embodiment, the relative abundance of one or more VFs or VPIs (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the degree or severity of Vascular

Dementia. In any of the aforesaid methods, detection of one or more VPIs described herein may optionally be combined with detection of one or more additional biomarkers for Vascular Dementia. Any suitable method in the art can be employed to measure the level of VFs and VPIs, including but not limited to the Preferred Technology described herein, kinase assays, immunoassays to detect and/or visualize the VPI (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.). In cases where a VPI has a known function, an assay for that function may be used to measure VPI expression. In a further embodiment, a decreased abundance of mRNA encoding one or more VPIs identified in Table IV (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the presence of Vascular Dementia. In yet a further embodiment, an increased abundance of mRNA encoding one or more VPIs identified in Table V (or any combination of them) in a test sample relative to a control sample or previously determined reference range indicates the presence of Vascular Dementia. Any suitable hybridization assay can be used to detect VPI expression by detecting and/or visualizing mRNA encoding the VPI (*e.g.*, Northern assays, dot blots, in situ hybridization, etc.).

In another embodiment of the invention, labelled antibodies, derivatives and analogs thereof, which specifically bind to a VPI can be used for diagnostic purposes to detect, diagnose, or monitor Vascular Dementia. Preferably, Vascular Dementia is detected in an animal, more preferably in a mammal and most preferably in a human.

5.13 Screening Assays

The invention provides methods for identifying agents (*e.g.*, candidate compounds or test compounds) that bind to a VPI or have a stimulatory or inhibitory effect on the expression or activity of a VPI. The invention also provides methods of identifying agents, candidate compounds or test compounds that bind to a VPI-related polypeptide or a VPI fusion protein or have a stimulatory or inhibitory effect on the expression or activity of a VPI-related polypeptide or a VPI fusion protein. Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules and other drugs. Agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one- compound" library method; and synthetic library methods using affinity chromatography selection. The

biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* (1997) 12:145; U.S. Patent No. 5,738,996; and U.S. Patent No.5,807,683, each of which is incorporated herein in its entirety by reference).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al, *Proc. Natl. Acad. Sci. USA* (1993) 90:6909; Erb et al, *Proc. Natl. Acad. Sci. USA* (1994) 91:11422; Zuckermann et al, *J. Med. Chem.* (1994) 37:2678; Cho et al, *Science* (1993) 261:1303; Carrell et al, *Angew. Chem. Int. Ed. Engl.* (1994) 33:2059; Carell et al, *Angew. Chem. Int. Ed. Engl.* (1994) 33:2061; and Gallop et al, *J. Med. Chem.* (1994) 37:1233, each of which is incorporated herein in its entirety by reference.

Libraries of compounds may be presented, *e.g.*, presented in solution (*e.g.*, Houghten, *Bio/Techniques* (1992) 13:412-421), or on beads (Lam, *Nature* (1991) 354:82-84), chips (Fodor, *Nature* (1993) 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al, *Proc. Natl. Acad. Sci. USA* (1992) 89:1865-1869) or phage (Scott and Smith, *Science* (1990) 249:386-390; Devlin, *Science* (1990) 249:404-406; Cwirla et al, *Proc. Natl. Acad. Sci. USA* (1990) 87:6378-6382; and Felici, *J. Mol. Biol.* (1990) 222:301- 310), each of which is incorporated herein in its entirety by reference.

In one embodiment, agents that interact with (*i.e.*, bind to) a VPI, a VPI fragment (*e.g.* a functionally active fragment), a VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein are identified in a cell-based assay system. In accordance with this embodiment, cells expressing a VPI, a fragment of a VPI, a VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein are contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the VPI is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. The cell, for example, can be of prokaryotic origin (*e.g.*, E. coli) or eukaryotic origin (*e.g.*, yeast or mammalian). Further, the cells can express the VPI, fragment of the VPI, VPI-related polypeptide, a fragment of the VPI-related polypeptide, or a VPI fusion protein endogenously or be genetically engineered to express the VPI, fragment of the VPI, VPI-related polypeptide, a fragment of the VPI- related polypeptide, or a VPI fusion protein. In certain instances, the VPI, fragment of the VPI, VPI-related polypeptide, a fragment of the VPI-related polypeptide, or a VPI fusion protein or the candidate compound is labelled, for example with a radioactive label (such as ^{32}P, ^{35}S or ^{125}I) or a fluorescent label (such as fluorescein isothiocyanate, rhodamine, phycoerythrin,

phycocyanin, allophycocyanin, o-phthaldehyde or fluorescamine) to enable detection of an interaction between a VPI and a candidate compound. The ability of the candidate compound to interact directly or indirectly with a VPI, a fragment of a VPI, a VPI-related polypeptide, a fragment of a VPI- related polypeptide, or a VPI fusion protein can be determined by methods known to those of skill in the art. For example, the interaction between a candidate compound and a VPI, a fragment of a VPI, a VPI-related polypeptide, a fragment of a VPI- related polypeptide, or a VPI fusion protein can be determined by flow cytometry, a scintillation assay, immunoprecipitation or western blot analysis.

In another embodiment, agents that interact with (*i.e.*, bind to) a VPI, a VPI fragment (*e.g.*, a functionally active fragment) a VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein are identified in a cell-free assay system. In accordance with this embodiment, a native or recombinant VPI or fragment thereof, or a native or recombinant VPI-related polypeptide or fragment thereof, or a VPI-fusion protein or fragment thereof, is contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the VPI or VPI-related polypeptide, or VPI fusion protein is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. Preferably, the VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI-fusion protein is first immobilized, by, for example, contacting the VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein with an immobilized antibody which specifically recognizes and binds it, or by contacting a purified preparation of the VPI, VPI fragment, VPI-related polypeptide, fragment of a VPI-related polypeptide, or a VPI fusion protein with a surface designed to bind proteins. The VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein may be partially or completely purified (*e.g.*, partially or completely free of other polypeptides) or part of a cell lysate. Further, the VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide may be a fusion protein comprising the VPI or a biologically active portion thereof, or VPI-related polypeptide and a domain such as glutathionine- S-transferase. Alternatively, the VPI, VPI fragment, VPI-related polypeptide, fragment of a VPI-related polypeptide or VPI fusion protein can be biotinylated using techniques well known to those of skill in the art (*e.g.*, biotinylation kit, Pierce Chemicals; Rockford, IL). The ability of the candidate compound to interact with a VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein can be can be determined by methods known to those of skill in the art.

In another embodiment, a cell-based assay system is used to identify agents that bind to or modulate the activity of a protein, such as an enzyme, or a biologically active portion thereof, which is responsible for the production or degradation of a VPI or is responsible for the post- translational modification of a VPI. In a primary screen, a plurality (*e.g.*, a library) of compounds are contacted with cells that naturally or recombinantly express: (i) a VPI, an isoform of a VPI, a VPI homolog a VPI-related polypeptide, a VPI fusion protein, or a biologically active fragment of any of the foregoing; and (ii) a protein that is responsible for processing of the VPI, VPI isoform, VPI homolog, VPI-related polypeptide, VPI fusion protein, or fragment in order to identify compounds that modulate the production, degradation, or post-translational modification of the VPI, VPI isoform, VPI homolog, VPI-related polypeptide, VPI fusion protein or fragment. If desired, compounds identified in the primary screen can then be assayed in a secondary screen against cells naturally or recombinantly expressing the specific VPI of interest. The ability of the candidate compound to modulate the production, degradation or post-translational modification of a VPI, isoform, homolog, VPI-related polypeptide, or VPI fusion protein can be determined by methods known to those of skill in the art, including without limitation, flow cytometry, a scintillation assay, immunoprecipitation and western blot analysis.

In another embodiment, agents that competitively interact with (*i.e.*, bind to) a VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein are identified in a competitive binding assay. In accordance with this embodiment, cells expressing a VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein are contacted with a candidate compound and a compound known to interact with the VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide or a VPI fusion protein; the ability of the candidate compound to competitively interact with the VPI, VPI fragment, VPI-related polypeptide, fragment of a VPI-related polypeptide, or a VPI fusion protein is then determined. Alternatively, agents that competitively interact with (*i.e.*, bind to) a VPI, VPI fragment, VPI-related polypeptide or fragment of a VPI-related polypeptide are identified in a cell-free assay system by contacting a VPI, VPI fragment, VPI-related polypeptide, fragment of a VPI-related polypeptide, or a VPI fusion protein with a candidate compound and a compound known to interact with the VPI, VPI-related polypeptide or VPI fusion protein. As stated above, the ability of the candidate compound to interact with a VPI, VPI fragment, VPI-related polypeptide, a fragment of a VPI-related polypeptide, or a VPI fusion protein can be

determined by methods known to those of skill in the art. These assays, whether cell-based or cell- free, can be used to screen a plurality (*e.g.*, a library) of candidate compounds.

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression of a VPI, or a VPI-related polypeptide are identified by contacting cells (*e.g.*, cells of prokaryotic origin or eukaryotic origin) expressing the VPI, or VPI- related polypeptide with a candidate compound or a control compound (*e.g.*, phosphate buffered saline (PBS)) and determining the expression of the VPI, VPI-related polypeptide, or VPI fusion protein, mRNA encoding the VPI, or mRNA encoding the VPI-related polypeptide. The level of expression of a selected VPI, VPI-related polypeptide, mRNA encoding the VPI, or mRNA encoding the VPI-related polypeptide in the presence of the candidate compound is compared to the level of expression of the VPI, VPI-related polypeptide, mRNA encoding the VPI, or mRNA encoding the VPI-related polypeptide in the absence of the candidate compound (*e.g.*, in the presence of a control compound). The candidate compound can then be identified as a modulator of the expression of the VPI, or a VPI-related polypeptide based on this comparison. For example, when expression of the VPI or mRNA is significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of expression of the VPI or mRNA. Alternatively, when expression of the VPI or mRNA is significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of the expression of the VPI or mRNA. The level of expression of a VPI or the mRNA that encodes it can be determined by methods known to those of skill in the art. For example, mRNA expression can be assessed by Northern blot analysis or RT-PCR, and protein levels can be assessed by western blot analysis.

In another embodiment, agents that modulate the activity of a VPI, or a VPI- related polypeptide are identified by contacting a preparation containing the VPI or VPI-related polypeptide, or cells (*e.g.*, prokaryotic or eukaryotic cells) expressing the VPI or VPI-related polypeptide with a test compound or a control compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the VPI or VPI-related polypeptide. The activity of a VPI or a VPI-related polypeptide can be assessed by detecting induction of a cellular signal transduction pathway of the VPI or VPI-related polypeptide (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the target on a suitable substrate, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a VPI or a VPI-related polypeptide and is operably linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cellular differentiation, or cell proliferation. Based on the present description,

techniques known to those of skill in the art can be used for measuring these activities (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference). The candidate compound can then be identified as a modulator of the activity of a VPI or VPI-related polypeptide by comparing the effects of the candidate compound to the control compound. Suitable control compounds include phosphate buffered saline (PBS) and normal saline (NS).

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression, activity or both the expression and activity of a VPI or VPI-related polypeptide are identified in an animal model. Examples of suitable animals include, but are not limited to, mice, rats, rabbits, monkeys, guinea pigs, dogs and cats. Preferably, the animal used represents a model of Vascular Dementia (*e.g.*, animal models of cerebral beta-amyloid angiopathy (Walker, *Brain Res. Rev.* (1997) 25:70-84), animal models of vascular dementia with emphasis on stroke-prone spontaneously hypertensive rats (Saito et al., *Clin. Exp. Pharmacol. Physiol.* (1995) 22 Suppl 1:s257-259), unilateral middle cerebral artery (MCA) occlusion, multiple small embolization or transient four-vessel occlusion model rats producing acute single or multi-infarct dementia memory impairments (Naritomi, *Alzheimer Dis Assoc Disord* (1991) 5:103-11), stroke-prone spontaneously hypertensive rats (Togashi et al., *Stroke* (1996) 27:520-5; discussion 525-6), Icelandic-like mutation of Cystatin C in an animal model of cerebrovascular beta-amyloidosis (Wei, *Stroke* (1996) 27:2080-5), and finally common models of stroke as reviewed by Feuerstein and Wang (Feuerstein GZ, Wang X, Animal models of stroke, *Mol Med Today* 2000 Mar;6(3):133-5)). In accordance with this embodiment, the test compound or a control compound is administered (*e.g.*, orally, rectally or parenterally such as intraperitoneally or intravenously) to a suitable animal and the effect on the expression, activity or both expression and activity of the VPI or VPI-related polypeptide is determined. Changes in the expression of a VPI or VPI-related polypeptide can be assessed by the methods outlined above.

In yet another embodiment, a VPI or VPI-related polypeptide is used as a "bait protein" in a two-hybrid assay or three hybrid assay to identify other proteins that bind to or interact with a VPI or VPI-related polypeptide (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos et al, *Cell* (1993) 72:223-232; Madura et al, *J. Biol. Chem.* (1993) 268:12046-12054; Bartel et al, *Bio/Techniques* (1993) 14:920-924; Iwabuchi et al, *Oncogene* (1993) 8:1693-1696; and PCT Publication No. WO 94/10300). As those skilled in the art will appreciate, such binding proteins are also likely to be involved in the propagation of signals by the VPIs of the invention as, for example, upstream or downstream elements of a signaling pathway involving the VPIs of the invention.

Table XI enumerates scientific publications describing suitable assays for detecting or quantifying enzymatic or binding activity of a VPI, a VPI analog, a VPI- related polypeptide, or a fragment of any of the foregoing. Each such reference is hereby incorporated in its entirety. In a preferred embodiment, as assay referenced in Table XI is used in the screens and assays described herein, for example to screen for or identify a compound that modulates the activity of (or that modulates both the expression and activity of) a VPI, VPI analog, or VPI-related polypeptide, a fragment of any of the foregoing.

Table XI.

VPI	References
VPI-25 VPI-88 VPI-99	Structural Biology 2000 7: 312-321, J. Am. Chem. Soc. 2000 122: 2178-2192,
VPI-113	Clin Chem 1993 Feb 39(2): 309-12 J Immunol Methods 1987 Aug 24 102:1 7-14
VPI-27 VPI-41 VPI-42 VPI-43	Neuroendocrinology 1992 Mar 55:3 308-16
VPI-48	J Chromatogr 1987 Dec 18 411: 498-501 Eisei Shikenjo Hokoku 1972 90: 89-92 Analyst 1990 Aug 115:8 1143-4

This invention further provides novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

5.14 Therapeutic Uses of VPIs

The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound. Such compounds include but are not limited to: VPIs, VPI analogs, VPI-related polypeptides and derivatives (including fragments) thereof; antibodies to the foregoing; nucleic acids encoding VPIs, VPI analogs, VPI-related polypeptides and fragments thereof; antisense nucleic acids to a gene encoding a VPI or VPI-related polypeptide; and modulator (*e.g.*, agonists and antagonists) of a gene encoding a VPI or VPI-related polypeptide. An important feature of the present invention is the identification of genes encoding VPIs involved in Vascular Dementia. Vascular Dementia can be treated (*e.g.* to ameliorate symptoms or to retard onset or progression) or prevented by administration of a therapeutic compound that promotes function or expression of one or more VPIs that are decreased in the CSF of Vascular Dementia subjects having Vascular Dementia, or by

administration of a therapeutic compound that reduces function or expression of one or more VPIs that are increased in the CSF of subjects having Vascular Dementia.

In one embodiment, one or more antibodies each specifically binding to a VPI are administered alone or in combination with one or more additional therapeutic compounds or treatments. Examples of such therapeutic compounds or treatments include, but are not limited to, antithrombic therapies such as Danaparoid, Nadroparin and Tinzaparin, thrombolytic and defibrinogenating agents such as Pro-urokinase, streptokinase, tissue plasmoinogen activator and urokinase, antiplatelet agents such as aspirin, Buflomedil (Cucinotta et al. *J Int Med Res* (1992) 20:136-49), neuroprotective agents such as Propentofylline (Rother et al. Ann *N Y Acad Sci* (1996) 777:404-9, Mielke et al. *Alzheimer Dis Assoc Disord* (1998) 12 Suppl 2:S29-35, Rother et al. *Dement Geriatr Cogn Disord* (1998) 9 Suppl 1:36-43), cholinesterase inhibitors such as rivastigmine, galantamine (Kumar et al. *Neurology* (1999) 52 Suppl 2:A395) and other cytoprotective agentscurrently under clinical evaluation such as the calcium antagonists Nimodipine andNicadipine, NMDA antagonists such as Selfotel, Dextrorphan, Cerestat, Eliprodil, Lamortigine, GABA agonists, Kappa-selective opiod antagonists, Lubeluzole, Free radicalscavengers, anti-ICAM antibodies and GM-1 ganglioside, Abbokinase®, Activase®, Aggrenox®, Anti-ICAM-1 antibody, Anti-beta-2-integrin antibody, Arvin®, Atacand®, CerAxon®, Cerebyx®, Ceresine®, Cerestat®, Cervene®, Coumadin®, Fiblast®,Fraxiparine®, Freedox®, Innohep®, Kabikinase®, Klerval®, LeukArrest®, Lipitor®, Lovenox®, Neurogard®, Nimotop®, Orgaran®, Persantine®, Plavix®, Prolyse®, Prosynap®, ReoPro®, Selfotel®, Sibelium®, Streptase®, Streptokinase, Sygen®, Ticlid®, Trental®, Viprinex®, Warfarin, Zanaflex®, Zendra®.

Preferably, a biological product such as an antibody is allogeneic to the subject to which it is administered. In a preferred embodiment, a human VPI or a human VPI- related polypeptide, a nucleotide sequence encoding a human VPI or a human VPI- related polypeptide, or an antibody to a human VPI or a human VPI-related polypeptide, is administered to a human subject for therapy (*e.g.* to ameliorate symptoms or to retard onset or progression) or prophylaxis.

5.14.1 Treatment And Prevention Of Vascular Dementia

Vascular Dementia is treated or prevented by administration to a subject suspected of having or known to have Vascular Dementia or to be at risk of developing Vascular Dementia of a compound that modulates (*i.e.*, increases or decreases) the level or activity (*i.e.*, function) of one or more VPIs or the level of one or more VFs that are differentially present in the CSF

of subjects having Vascular Dementia compared with CSF of subjects free from Vascular Dementia. In one embodiment, Vascular Dementia is treated or prevented by administering to a subject suspected of having or known to have Vascular Dementia or to be at risk of developing Vascular Dementia a compound that upregulates (*i.e.*, increases) the level or activity (*i.e.*, function) of one or more VPIs or the level of one or more VFs that are decreased in the CSF of subjects having Vascular Dementia. In another embodiment, a compound is administered that downregulates the level or activity (*i.e.*, function) of one or more VPIs or the level of one or more VFs that are increased in the CSF of subjects having Vascular Dementia. Examples of such a compound include but are not limited to: VPIs, VPI fragments and VPI-related polypeptides; nucleic acids encoding a VPI, a VPI fragment and a VPI-related polypeptide (*e.g.*, for use in gene therapy); and, for those VPIs or VPI-related polypeptides with enzymatic activity, compounds or molecules known to modulate that enzymatic activity. Other compounds that can be used, *e.g.*, VPI agonists, can be identified using *in vitro* assays.

Vascular Dementia is also treated or prevented by administration to a subject suspected of having or known to have Vascular Dementia or to be at risk of developing Vascular Dementia of a compound that downregulates the level or activity of one or more VPIs or the level of one or more VFs that are increased in the CSF of subjects having Vascular Dementia. In another embodiment, a compound is administered that upregulates the level or activity of one or more VPIs or the level of one or more VFs that are decreased in the CSF of subjects having Vascular Dementia. Examples of such a compound include, but are not limited to, VPI antisense oligonucleotides, ribozymes, antibodies directed against VPIs, and compounds that inhibit the enzymatic activity of a VPI. Other useful compounds *e.g.*, VPI antagonists and small molecule VPI antagonists, can be identified using *in vitro* assays.

In a preferred embodiment, therapy or prophylaxis is tailored to the needs of an individual subject. Thus, in specific embodiments, compounds that promote the level or function of one or more VPIs, or the level of one or more VFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have Vascular Dementia, in whom the levels or functions of said one or more VPIs, or levels of said one or more VFs, are absent or are decreased relative to a control or normal reference range. In further embodiments, compounds that promote the level or function of one or more VPIs, or the level of one or more VFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have Vascular Dementia in whom the levels or functions of said one or more VPIs, or levels of said one or more VFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function

of one or more VPIs, or the level of one or more VFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have Vascular Dementia in whom the levels or functions of said one or more VPIs, or levels of said one or more VFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function of one or more VPIs, or the level of one or more VFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have Vascular Dementia in whom the levels or functions of said one or more VPIs, or levels of said one or more VFs, are decreased relative to a control or to a reference range. The change in VPI function or level, or VF level, due to the administration of such compounds can be readily detected, *e.g.*, by obtaining a sample (*e.g.*, a sample of CSF, blood or urine or a tissue sample such as biopsy tissue) and assaying *in vitro* the levels of said VFs or the levels or activities of said VPIs, or the levels of mRNAs encoding said VPIs. or any combination of the foregoing. Such assays can be performed before and after the administration of the compound as described herein.

The compounds of the invention include but are not limited to any compound, *e.g.*, a small organic molecule, protein, peptide, antibody, nucleic acid, etc. that restores the Vascular Dementia VPI or VF profile towards normal with the proviso that such compounds do not include antithrombic therapies such as Danaparoid, Nadroparin and Tinzaparin, thrombolytic and defibrinogenating agents such as Pro-urokinase, streptokinase, tissue plasmoinogen activator and urokinase, antiplatelet agents such as aspirin, Buflomedil (Cucinotta et al. *J Int Med Res* (1992) 20:136-49), neuroprotective agents such as Propentofylline (Rother et al. Ann *N Y Acad Sci* (1996) 777:404-9, Mielke et al. *Alzheimer Dis Assoc Disord* (1998) 12 Suppl 2:S29-35, Rother et al. *Dement Geriatr Cogn Disord* (1998) 9 Suppl 1:36-43), cholinesterase inhibitors such as rivastigmine, galantamine (Kumar et al. *Neurology* (1999) 52 Suppl 2:A395) and other cytoprotective agentscurrently under clinical evaluation such as the calcium antagonists Nimodipine andNicadipine, NMDA antagonists such as Selfotel, Dextrorphan, Cerestat, Eliprodil, Lamortigine, GABA agonists, Kappa-selective opiod antagonists, Lubeluzole, Free radicalscavengers, anti-ICAM antibodies and GM-1 ganglioside, Abbokinase®, Activase®, Aggrenox®, Anti-ICAM-1 antibody, Anti-beta-2-integrin antibody, Arvin®, Atacand®, CerAxon®, Cerebyx®, Ceresine®, Cerestat®, Cervene®, Coumadin®, Fiblast®,Fraxiparine®, Freedox®, Innohep®, Kabikinase®, Klerval®, LeukArrest®, Lipitor®, Lovenox®, Neurogard®, Nimotop®, Orgaran®, Persantine®, Plavix®, Prolyse®, Prosynap®, ReoPro®, Selfotel®, Sibelium®, Streptase®, Streptokinase, Sygen®, Ticlid®, Trental®, Viprinex®, Warfarin, Zanaflex®, Zendra®.

5.14.2 Gene Therapy

In a specific embodiment, nucleic acids comprising a sequence encoding a VPI, a VPI fragment, VPI-related polypeptide or fragment of a VPI-related polypeptide, are administered to promote VPI function by way of gene therapy. Gene therapy refers to administration to a subject of an expressed or expressible nucleic acid. In this embodiment, the nucleic acid produces its encoded polypeptide that mediates a therapeutic effect by promoting VPI function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al, *Clinical Pharmacy* (1993) 12:488-505; Wu and Wu, *Biotherapy* (1991) 3:87-95; Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.* (1993) 32:573-596; Mulligan, *Science* (1993) 260:926- 932; and Morgan and Anderson, *Ann. Rev. Biochem.* (1993) 62:191-217; May, 1993, TIBTECH 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al, (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the compound comprises a nucleic acid encoding a VPI or fragment or chimeric protein thereof, said nucleic acid being part of an expression vector that expresses a VPI or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the VPI coding region, said promoter being inducible or constitutive (and, optionally, tissue-specific). In another particular embodiment, a nucleic acid molecule is used in which the VPI coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the VPI nucleic acid (Koller and Smithies, *Proc. Natl. Acad. Sci. USA* (1989) 86:8932-8935; Zijlstra et al, *Nature* (1989) 342:435-438).

Delivery of the nucleic acid into a subject may be direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid-carrying vector; this approach is known as *in vivo* gene therapy. Alternatively, delivery of the nucleic acid into the subject may be indirect, in which case cells are first transformed with the nucleic acid *in vitro* and then transplanted into the subject; this approach is known as ex vivo gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286); by direct injection of naked DNA; by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); by coating with lipids, cell-surface receptors or transfecting agents; by encapsulation in liposomes, microparticles or microcapsules; by administering it in linkage to a peptide which is known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, *J. Biol. Chem.* (1987) 262:4429- 4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al,); WO 92/22635 dated December 23, 1992 (Wilson et al,); WO92/20316 dated November 26, 1992 (Findeis et al,); WO93/14188 dated July 22, 1993 (Clarke et al,), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al, *Nature* (1989) 342:435-438).

In a specific embodiment, a viral vector that contains a nucleic acid encoding a VPI is used. For example, a retroviral vector can be used (see Miller et al, *Meth. Enzymol.* (1993) 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid encoding the VPI to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a subject. More detail about retroviral vectors can be found in Boesen et al, *Biotherapy* (1994) 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al, *J. Clin. Invest.* (1994) 93:644-651; Kiem et al, *Blood* (1994) 83:1467-1473; Salmons and Gunzberg, *Human Gene Therapy* (1993) 4:129-141; and Grossman and Wilson, *Curr. Opin. in Genetics and Devel.* (1993) 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, *Current Opinion in Genetics and Development* (1993) 3:499-503 present a review of adenovirus-based gene therapy. Bout et al, *Human Gene Therapy* (1994) 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al, *Science* (1991) 252:431-434; Rosenfeld et al, *Cell* (1992) 68:143-155; Mastrangeli et al, *J. Clin. Invest.* (1993) 91:225-234; PCT Publication WO94/12649; and Wang, et al, *Gene Therapy* (1995) 2:775-783.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al, *Proc. Soc. Exp. Biol. Med.* (1993) 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, *Meth. Enzymol.* (1993) 217:599-618; Cohen et al, *Meth. Enzymol.* (1993) 217:618-644; Cline, *Pharmac. Ther.* (1985) 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a subject by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the subject.

Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, the condition of the subject, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to neuronal cells, glial cells (*e.g.*, oligodendrocytes or astrocytes), epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood or fetal liver.

In a preferred embodiment, the cell used for gene therapy is autologous to the subject that is treated.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding a VPI is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem or progenitor cells which can be isolated and maintained *in vitro* can be used in accordance with this embodiment of the present invention (see *e.g.* PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, *Meth. Cell Bio.* (1980) 21A:229; and Pittelkow and Scott, *Mayo Clinic Proc.* (1986) 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Direct injection of a DNA coding for a VPI may also be performed according to, for example, the techniques described in United States Patent No. 5,589,466. These techniques involve the injection of "naked DNA", *i.e.*, isolated DNA molecules in the absence of liposomes, cells, or any other material besides a suitable carrier. The injection of DNA encoding a protein and operably linked to a suitable promoter results in the production of the protein in cells near the site of injection and the elicitation of an immune response in the subject to the protein encoded by the injected DNA. In a preferred embodiment, naked DNA comprising (a) DNA encoding a VPI and (b) a promoter are injected into a subject to elicit an immune response to the VPI.

5.14.3 Inhibition of VPIs to Treat Vascular Dementia

In one embodiment of the invention, Vascular Dementia is treated or prevented by administration of a compound that antagonizes (inhibits) the level(s) and/or function(s) of one or more VPIs which are elevated in the CSF of subjects having Vascular Dementia as compared with CSF of subjects free from Vascular Dementia. Compounds useful for this purpose include but are not limited to anti-VPI antibodies (and fragments and derivatives containing the binding region thereof), VPI antisense or ribozyme nucleic acids, and nucleic acids encoding dysfunctional VPIs that are used to "knockout" endogenous VPI function by homologous recombination (see, *e.g.*, Capecchi, *Science* (1989) 244:1288-1292). Other compounds that inhibit VPI function can be identified by use of known *in vitro* assays, *e.g.*, assays for the ability of a test compound to inhibit binding of a VPI to another protein or a binding partner, or to inhibit a known VPI function. Preferably such inhibition is assayed *in vitro* or in cell culture, but genetic assays may also be employed. The Preferred Technology can also be used to detect levels of the VPI before and after the administration of the compound. Preferably, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific compound and whether its administration is indicated for treatment of the affected tissue, as described in more detail below.

In a specific embodiment, a compound that inhibits a VPI function is administered therapeutically or prophylactically to a subject in whom an increased CSF level or functional activity of the VPI (*e.g.*, greater than the normal level or desired level) is detected as compared with CSF of subjects free from Vascular Dementia or a predetermined reference range. Methods standard in the art can be employed to measure the increase in a VPI level c function, as outlined above. Preferred VPI inhibitor compositions include small molecules, *i.e.*, molecules of 1000 daltons or less. Such small molecules can be identified by the screening methods described herein.

5.14.4 Antisense Regulation of VPIs

In a specific embodiment, VPI expression is inhibited by use of VPI antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least six nucleotides that are antisense to a gene or cDNA encoding a VPI or a portion thereof. As used herein, a VPI "antisense" nucleic acid refers to a nucleic acid capable of hybridizing by virtue of some sequence complementarity to a portion of an RNA (preferably mRNA) encoding a VPI. The antisense nucleic acid may be complementary to a coding and/or noncoding region of an mRNA encoding a VPI. Such antisense nucleic acids

have utility as compounds that inhibit VPI expression, and can be used in the treatment or prevention of Vascular Dementia.

The antisense nucleic acids of the invention are double-stranded or single- stranded oligonucleotides, RNA or DNA or a modification or derivative thereof, and can be directly administered to a cell or produced intracellularly by transcription of exogenous, introduced sequences.

The invention further provides pharmaceutical compositions comprising an effective amount of the VPI antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention provides methods for inhibiting the expression of a VPI nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising a VPI antisense nucleic acid of the invention.

VPI antisense nucleic acids and their uses are described in detail below.

5.14.5 VPI Antisense Nucleic Acids

The VPI antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides ranging from 6 to about 50 oligonucleotides. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof and can be single- stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appended groups such as peptides; agents that facilitate transport across the cell membrane (see, *e.g.*, Letsinger et al, *Proc. Natl. Acad. Sci. USA* (1989) 86:6553-6556; Lemaitre et al, *Proc. Natl. Acad. Sci. USA* (1987) 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988); hybridization-triggered cleavage agents (see, *e.g.*, Krol et al, *BioTechniques* (1988) 6:958-976) or intercalating agents (see, *e.g.*, Zon, *Pharm. Res.* (1988) 5:539-549).

In a preferred aspect of the invention, a VPI antisense oligonucleotide is provided, preferably of single-stranded DNA. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The VPI antisense oligonucleotide may comprise at least one of the following modified base moieties: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil,

hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta- D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine, and other base analogs.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, *e.g.*, one of the following sugar moieties: arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one of the following modified phosphate backbones: a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, a formacetal, or an analog of formacetal.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al, 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al, (*Nucl. Acids Res.* (1988) 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al, *Proc. Natl. Acad. Sci. USA* (1988) 85:7448-7451).

In a specific embodiment, the VPI antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion

thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the VPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the VPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Examples of such promoters are outlined above.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a gene encoding a VPI, preferably a human gene encoding a VPI. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize under stringent conditions (*e.g.*, highly stringent conditions comprising hybridization in 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65 °C and washing in 0.1xSSC/0.1% SDS at 68 °C, or moderately stringent conditions comprising washing in 0.2xSSC/0.1% SDS at 42 °C) with the RNA, forming a stable duplex; in the case of double-stranded VPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding a VPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.14.6 Therapeutic Use of VPI Antisense Nucleic Acids

The VPI antisense nucleic acids can be used to treat or prevent Vascular Dementia when the target VPI is overexpressed in the CSF of subjects suspected of having or suffering from Vascular Dementia. In a preferred embodiment, a single-stranded DNA antisense VPI oligonucleotide is used.

Cell types which express or overexpress RNA encoding a VPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, astrocytes, glial cells,

neuronal cells, and ependymal cells). Such methods include, but are not limited to, hybridization with a VPI-specific nucleic acid (*e.g.*, by Northern hybridization, dot blot hybridization, in situ hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into a VPI, immunoassay, etc.. In a preferred aspect, primary tissue from a subject can be assayed for VPI expression prior to treatment, *e.g.*, by immunocytochemistry or in situ hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of a VPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a subject having Vascular Dementia.

The amount of VPI antisense nucleic acid which will be effective in the treatment of Vascular Dementia can be determined by standard clinical techniques.

In a specific embodiment, pharmaceutical compositions comprising one or more VPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, such compositions may be used to achieve sustained release of the VPI antisense nucleic acids.

5.14.7 Inhibitory Ribozyme andTriple Helix Approaches

In another embodiment, symptoms of Vascular Dementia may be ameliorated by decreasing the level of a VPI or VPI activity by using gene sequences encoding the VPI in conjunction with well-known gene "knock-out," ribozyme or triple helix methods to decrease gene expression of a VPI. In this approach ribozyme or triple helix molecules are used to modulate the activity, expression or synthesis of the gene encoding the VPI, and thus to ameliorate the symptoms of Vascular Dementia. Such molecules may be designed to reduce or inhibit expression of a mutant or non-mutant target gene. Techniques for the production and use of such molecules are well known to those of skill in the art.

Ribozyme molecules designed to catalytically cleave gene mRNA transcripts encoding a VPI can be used to prevent translation of target gene mRNA and, therefore, expression of the gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver et al, *Science* (1990) 247:1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, *Current Biology* (1994) 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene

mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding a VPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5′- UG-3′. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585- 591, each of which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5′ end of the mRNA encoding the VPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, et al, *Science*, (1984) 224:574-578; Zaug and Cech, *Science*, (1986) 231, 470-475; Zaug, et al, *Nature*, (1986) 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, *Cell*, (1986) 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the VPI.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the VPI *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the VPI and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficacy.

Endogenous VPI expression can also be reduced by inactivating or "knocking out" the gene encoding the VPI, or the promoter of such a gene, using targeted homologous

recombination (*e.g.*, see Smithies, et al, *Nature* (1985) 317:230-234; Thomas and Capecchi, *Cell* (1987) 51:503-512; Thompson et al, *Cell* (1989) 5:313-321; and Zijlstra et al, *Nature* (1989) 342:435-438, each of which is incorporated by reference herein in its entirety). For example, a mutant gene encoding a non-functional VPI (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene encoding the VPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, supra). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, the endogenous expression of a gene encoding a VPI can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene encoding the VPI in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6):569-584; Helene, et al, *Ann. N.Y. Acad. Sci.,* (1992) 660:27-36; and Maher, *Bioassays* (1992) 14(12):807- 815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) or translation (antisense, ribozyme) of mRNA produced by normal gene alleles of a VPI that the situation may arise wherein the concentration of VPI present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of activity of a gene encoding a VPI are maintained, gene therapy may be used to introduce into cells nucleic acid molecules that encode and express the VPI that exhibit normal gene activity and that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the gene encodes an extracellular protein, normal VPI can be co- administered in order to maintain the requisite level of VPI activity.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.15 Assays For Therapeutic Or Prophylactic Compounds

The present invention also provides assays for use in drug discovery in order to identify or verify the efficacy of compounds for treatment or prevention of Vascular Dementia. Test compounds can be assayed for their ability to restore VF or VPI levels in a subject having Vascular Dementia towards levels found in subjects free from Vascular Dementia or to produce similar changes in experimental animal models of Vascular Dementia. Compounds able to restore VF or VPI levels in a subject having Vascular Dementia towards

levels found in subjects free from Vascular Dementia or to produce similar changes in experimental animal models of Vascular Dementia can be used as lead compounds for further drug discovery, or used therapeutically. VF and VPI expression can be assayed by the Preferred Technology, immunoassays, gel electrophoresis followed by visualization, detection of VPI activity, or any other method taught herein or known to those skilled in the art. Such assays can be used to screen candidate drugs, in clinical monitoring or in drug development, where abundance of a VF or VPI can serve as a surrogate marker for clinical disease.

In various specific embodiments, *in vitro* assays can be carried out with cells representative of cell types involved in a subject's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used. Examples of animal models of Vascular Dementia include, but are not limited to, animal models of cerebral beta-amyloid angiopathy (Walker, *Brain Res. Rev.* (1997) 25:70-84), animal models of vascular dementia with emphasis on stroke-prone spontaneously hypertensive rats (Saito et al., *Clin. Exp. Pharmacol. Physiol.* (1995) 22 Suppl 1:s257-259), unilateral middle cerebral artery (MCA) occlusion, multiple small embolization or transient four-vessel occlusion model rats producing acute single or multi-infarct dementia memory impairments (Naritomi, *Alzheimer Dis Assoc Disord* (1991) 5:103-11), stroke-prone spontaneously hypertensive rats (Togashi et al., *Stroke* (1996) 27:520-5; discussion 525-6; Icelandic-like mutation of Cystatin C in an animal model of cerebrovascular beta-amyloidosis (Wei, *Stroke* (1996) 27:2080-5). Moreover, common models of stroke have been reviewed by Feuerstein and Wang (Feuerstein GZ, Wang X, Animal models of stroke, *Mol Med Today* 2000 Mar;6(3):133-5). Such animal models can be utilized to test compounds that modulate VF or VPI levels, since the neuropathology exhibited in these models is similar to that of Vascular Dementia. It is also apparent to the skilled artisan that, based upon the present disclosure, transgenic animals can be produced with "knock-out" mutations of the gene or genes encoding one or more VPIs. A "knock-out" mutation of a gene is a mutation that causes the mutated gene to not be expressed, or expressed in an aberrant form or at a low level, such that the activity associated with the gene product is nearly or entirely absent. Preferably, the transgenic animal is a mammal, more preferably, the transgenic animal is a mouse.

In one embodiment, test compounds that modulate the expression of a VPI are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for Vascular Dementia, expressing the VPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of the test compound on expression of one or more VPIs is determined. A test compound that alters the expression of a VPI (or a plurality of VPIs) can be identified by comparing the level of the selected VPI or VPIs (or mRNA(s) encoding the same) in an animal or group of animals treated with a test compound with the level of the VPI(s) or mRNA(s) in an animal or group of animals treated with a control compound. Techniques known to those of skill in the art can be used to determine the mRNA and protein levels, for example, in situ hybridization. The animals may or may not be sacrificed to assay the effects of a test compound.

In another embodiment, test compounds that modulate the activity of a VPI or a biologically active portion thereof are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for Vascular Dementia, expressing the VPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of a test compound on the activity of a VPI is determined. A test compound that alters the activity of a VPI (or a plurality of VPIs) can be identified by assaying animals treated with a control compound and animals treated with the test compound. The activity of the VPI can be assessed by detecting induction of a cellular second messenger of the VPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the VPI or binding partner thereof, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a VPI of the invention operably linked to a nucleic acid encoding a detectable marker, such as luciferase or green fluorescent protein), or detecting a cellular response (*e.g.*, cellular differentiation or cell proliferation). Techniques known to those of skill in the art can be utilized to detect changes in the activity of a VPI (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference).

In yet another embodiment, test compounds that modulate the level or expression of a VPI (or plurality of VPIs) are identified in human subjects having Vascular Dementia, preferably those having Vascular Dementia and most preferably those having severe Vascular Dementia. In accordance with this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on VPI expression is determined by analyzing the expression of the VPI or the mRNA encoding the same in a

biological sample (*e.g.*, CSF, serum, plasma, or urine). A test compound that alters the expression of a VPI can be identified by comparing the level of the VPI or mRNA encoding the same in a subject or group of subjects treated with a control compound to that in a subject or group of subjects treated with a test compound. Alternatively, alterations in the expression of a VPI can be identified by comparing the level of the VPI or mRNA encoding the same in a subject or group of subjects before and after the administration of a test compound. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression. For example, the Preferred Technology described herein can be used to assess changes in the level of a VPI.

In another embodiment, test compounds that modulate the activity of a VPI (or plurality of VPIs) are identified in human subjects having Vascular Dementia, preferably those having Vascular Dementia and most preferably those with severe Vascular Dementia. In this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on the activity of a VPI is determined. A test compound that alters the activity of a VPI can be identified by comparing biological samples from subjects treated with a control compound to samples from subjects treated with the test compound. Alternatively, alterations in the activity of a VPI can be identified by comparing the activity of a VPI in a subject or group of subjects before and after the administration of a test compound. The activity of the VPI can be assessed by detecting in a biological sample (*e.g.*, CSF, serum, plasma, or urine) induction of a cellular signal transduction pathway of the VPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), catalytic or enzymatic activity of the VPI or a binding partner thereof, or a cellular response, for example, cellular differentiation, or cel proliferation. Techniques known to those of skill in the art can be used to detect changes in the induction of a second messenger of a VPI or changes in a cellular response. For example, RT-PCR can be used to detect changes in the induction of a cellular second messenger.

In a preferred embodiment, a test compound that changes the level or expression of a VPI towards levels detected in control subjects (*e.g.*, humans free from Vascular Dementia) is selected for further testing or therapeutic use. In another preferred embodime[illegible], a test compound that changes the activity of a VPI towards the activity found in contro. subjects (*e.g.*, humans free from Vascular Dementia) is selected for further testing or therapeutic use.

In another embodiment, test compounds that reduce the severity of one or more symptoms associated with Vascular Dementia are identified in human subjects having Vascular Dementia, preferably subjects having Vascular Dementia and most preferably subjects with severe Vascular Dementia. In accordance with this embodiment, a test

compound or a control compound is administered to the subjects, and the effect of a test compound on one or more symptoms of Vascular Dementia is determined. A test compound that reduces one or more symptoms can be identified by comparing the subjects treated with a control compound to the subjects treated with the test compound. Techniques known to physicians familiar with Vascular Dementia can be used to determine whether a test compound reduces one or more symptoms associated with Vascular Dementia. For example, a test compound that enhances memory or reduces confusion in a subject having Vascular Dementia will be beneficial for treating subjects having Vascular Dementia.

In a preferred embodiment, a test compound that reduces the severity of one or more symptoms associated with Vascular Dementia in a human having Vascular Dementia is selected for further testing or therapeutic use.

5.16 Therapeutic and Prophylactic Compositions and Their Use

The invention provides methods of treatment (and prophylaxis) comprising administering to a subject an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration are described below.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, *J. Biol. Chem.* (1987) 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the

central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection into CSF or at the site (or former site) of neurodegeneration or to CNS tissue.

In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer, *Science* (1990) 249:1527-1533; Treat et al, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al, 1980, Surgery 88:507; Saudek et al, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., *Macromol. Sci. Rev. Macromol. Chem.* (1983) 23:61; see also Levy et al, *Science* (1985) 228:190; During et al, *Ann. Neurol.* (1989) 25:351; Howard et al, *J. Neurosurg.* (1989) 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* (1990) 249:1527-1533).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S.

Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al, *Proc. Natl. Acad. Sci. USA* (1991) 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic

aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of Vascular Dementia can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20- 500 micrograms of active compound pe kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

6. EXAMPLE: IDENTIFICATION OF PROTEINS DIFFERENTIALLY EXPRESSED IN THE CSF IN VASCULAR DEMENTIA

Using the following procedure, proteins in CSF samples from 5 subjects having Vascular Dementia and 5 control subjects were separated by isoelectric focusing followed by SDS-PAGE and analyzed. Parts 6.1.1 to 6.1.14 (inclusive) of the procedure set forth are hereby designated as the "Reference Protocol"

6.1 MATERIALS AND METHODS

6.1.1 Sample Preparation

A protein assay (Pierce BCA Cat # 23225) was performed on each CSF sample as received. Prior to protein separation, each sample was processed for selective depletion of certain proteins, in order to enhance and simplify protein separation and facilitate analysis by removing proteins that may interfere with or limit analysis of proteins of interest. See International Patent Application No. PCT/GB99/01742, filed June 1, 1999, which is incorporated by reference in its entirety, with particular reference to pages 3 and 6.

Removal of albumin, haptoglobin, transferrin and immunoglobin G (IgG) from CSF ("CSF depletion") was achieved by an affinity chromatography purification step in which the sample was passed through a series of 'Hi-Trap' columns containing immobilized antibodies for selective removal of albumin, haptoglobin and transferrin, and protein G for selective removal of immunoglobin G. Two affinity columns in a tandem assembly were prepared by coupling antibodies to protein G-sepharose contained in Hi-Trap columns (Protein G-Sepharose Hi-Trap columns (1 ml) Pharmacia Cat. No. 17-0404-01). This was done by circulating the following solutions sequentially through the columns: (1) Dulbecco's Phosphate Buffered Saline (Gibco BRL Cat. No. 14190-094); (2) concentrated antibody solution; (3) 200 mM sodium carbonate buffer, pH 8.35; (4) cross-linking solution (200 mM sodium carbonate buffer, pH 8.35, 20 mM dimethylpimelimidate); and (5) 500 mM ethanolamine, 500 mM NaCl. A third (un-derivatised) protein G Hi-Trap column was then attached to the lower end of the tandem column assembly.

The chromatographic procedure was automated using an Akta Fast Protein Liquid Chromatography (FPLC) System such that a series of up to seven runs could be performed sequentially. The samples were passed through the series of 3 Hi-Trap columns in which the affinity chromatography media selectively bind the above proteins thereby removing them from the sample. Fractions (typically 3 ml per tube) were collected of unbound material

("Flowthrough fractions") that eluted through the column during column loading and washing stages and of bound proteins ("Bound/Eluted fractions") that were eluted by step elution with Immunopure Gentle Ag/Ab Elution Buffer (Pierce Cat. No. 21013). The eluate containing unbound material was collected in fractions which were pooled, desalted/concentrated by centrifugal ultrafiltration and stored to await further analysis by 2D PAGE.

A volume of depleted CSF containing approximately 100-150 µg of total protein was aliquoted and an equal volume of 10% (w/v) SDS (Fluka 71729), 2.3% (w/v) dithiothreitol (BDH 443852A) was added. The sample was heated at 95 °C for 5 mins, and then allowed to cool to 20 °C . 125µl of the following buffer was then added to the sample:

8M urea (BDH 452043w)

4% CHAPS (Sigma C3023)

65mM dithiotheitol (DTT)

2% (v/v) Resolytes 3.5-10 (BDH 44338 2x) This mixture was vortexed, and centrifuged at 13000 rpm for 5 mins at 15 °C, and the supernatant was analyzed by isoelectric focusing.

6.1.2 Isoelectric Focusing

Isoelectric focusing (IEF), was performed using the Immobiline® DryStrip Kit (Pharmacia BioTech), following the procedure described in the manufacturer's instructions, see Instructions for Immobiline® DryStrip Kit, Pharmacia, # 18-1038-63, Edition AB (incorporated herein by reference in its entirety). Immobilized pH Gradient (IPG) strips (18cm, pH 3-10 non-linear strips; Pharmacia Cat. # 17-1235-01) were rehydrated overnight a 20 °C in a solution of 8M urea, 2% (w/v) CHAPS, 10mM DTT, 2% (v/v) Resolytes 3.5-10, as described in the Immobiline DryStrip Users Manual. For IEF, 50 µl of supernatant (prepared as above) was loaded onto a strip, with the cup-loading units being placed at the basic end of the strip. The loaded gels were then covered with mineral oil (Pharmacia 17-3335-01) and a voltage was immediately applied to the strips according to the following profile, using a Pharmacia EPS3500XL power supply (Cat 19-3500-01):

Initial voltage = 300V for 2 hrs

Linear Ramp from 300V to 3500V over 3hrs

Hold at 3500V for 19hrs For all stages of the process, the current limit was set to 10mA for 12 gels, and the wattage limit to 5W. The temperature was held at 20 °C throughout the run.

6.1.3 Gel Equilibration and SDS-PAGE

After the final 19hr step, the strips were immediately removed and immersed for 10 mins at 20 °C in a first solution of the following composition: 6M urea; 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol (Fluka 49767); 0.05M Tris/HCl, pH 6.8 (Sigma Cat T-1503). The strips were removed from the first solution and immersed for 10 mins at 20 °C in a second solution of the following composition: 6M urea; 2% (w/v) iodoacetamide (Sigma I-6125); 2% (w/v) SDS; 30% (v/v) glycerol; 0.05M Tris/HCl, pH 6.8. After removal from the second solution, the strips were loaded onto supported gels for SDS-PAGE according to Hochstrasser et al, 1988, Analytical Biochemistry 173: 412-423 (incorporated herein by reference in its entirety), with modifications as specified below.

6.1.4 Preparation of supported gels

The gels were cast between two glass plates of the following dimensions: 23cm wide x 24cm long (back plate); 23cm wide x 24cm long with a 2cm deep notch in the central 19cm (front plate). To promote covalent attachment of SDS-PAGE gels, the back plate was treated with a 0.4% solution of γ-methacryl-oxypropyltrimethoxysilane in ethanol (BindSilane™; Pharmacia Cat. # 17-1330-01). The front plate was treated with (RepelSilane™ Pharmacia Cat. # 17-1332-01) to reduce adhesion of the gel. Excess reagent was removed by washing with water, and the plates were allowed to dry. At this stage, both as identification for the gel, and as a marker to identify the coated face of the plate, an adhesive bar-code was attached to the back plate in a position such that it would not come into contact with the gel matrix.

The dried plates were assembled into a casting box with a capacity of 13 gel sandwiches. The top and bottom plates of each sandwich were spaced by means of 1mm thick spacers, 2.5 cm wide. The sandwiches were interleaved with acetate sheets to facilitate separation of the sandwiches after gel polymerization. Casting was then carried out according to Hochstrasser et al, op. cit.

A 9-16% linear polyacrylamide gradient was cast, extending up to a point 2cm below the level of the notch in the front plate, using the Angelique gradient casting system (Large Scale Biology). Stock solutions were as follows. Acrylamide (40% in water) was from Serva (Cat. # 10677). The cross-linking agent was PDA (BioRad 161-0202), at a concentration of 2.6% (w/w) of the total starting monomer content. The gel buffer was 0.375M Tris/HCl, pH 8.8. The polymerization catalyst was 0.05% (v/v) TEMED (BioRad 161-0801), and the initiator was 0.1% (w/v) APS (BioRad 161- 0700). No SDS was included in the gel and no

stacking gel was used. The cast gels were allowed to polymerize at 20 °C overnight, and then stored at 4 °C in sealed polyethylene bags with 6ml of gel buffer, and were used within 4 weeks.

6.1.5 SDS-PAGE

A solution of 0.5% (w/v) agarose (Fluka Cat 05075) was prepared in running buffer (0.025M Tris, 0.198M glycine (Fluka 50050), 1% (w/v) SDS, supplemented by a trace of bromophenol blue). The agarose suspension was heated to 70 °C with stirring, until the agarose had dissolved. The top of the supported 2nd D gel was filled with the agarose solution, and the equilibrated strip was placed into the agarose, and tapped gently with a palette knife until the gel was intimately in contact with the 2nd D gel. The gels were placed in the 2nd D running tank, as described by Amess et al, 1995, Electrophoresis 16: 1255-1267 (incorporated herein by reference in its entirety). The tank was filled with running buffer (as above) until the level of the buffer was just higher than the top of the region of the 2nd D gels which contained polyacrylamide, so as to achieve efficient cooling of the active gel area. Running buffer was added to the top buffer compartments formed by the gels, and then voltage was applied immediately to the gels using a Consort E-833 power supply. For 1 hour, the gels were run at 20mA/gel. The wattage limit was set to 150W for a tank containing 6 gels, and the voltage limit was set to 600V. After 1 hour, the gels were then run at 40mA/gel, with the same voltage and wattage limits as before, until the bromophenol blue line was 0.5cm from the bottom of the gel. The temperature of the buffer was held at 16 °C throughout the run. Gels were not run in duplicate.

6.1.6 Staining

Upon completion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol (BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ- Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were stained by complete immersion for 4 hours in a staining solution of Pyridinium, 4-[2-[4-(dipentylamino)-2- trifluoromethylphenyl]

ethenyl]-1-(sulfobutyl)-, inner salt, prepared by diluting a stock solution of this dye (2mg/ml in DMSO) in 7.5% (v/v) aqueous acetic acid to give a final concentration of 1.2 mg/l; the staining solution was vacuum filtered through a 0.4μm filter (Duropore) before use.

6.1.7 Imaging of the gel

A computer-readable output was produced by imaging the fluorescently stained gels with the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK) described in section 5.1, supra. This scanner has a gel carrier with four integral fluorescent markers (Designated M1, M2, M3, M4) that are used to correct the image geometry and are a quality control feature to confirm that the scanning has been performed correctly.

For scanning, the gels were removed from the stain, rinsed with water and allowed to air dry briefly, and imaged on the Apollo 2. After imaging, the gels were sealed in polyethylene bags containing a small volume of staining solution, and then stored at 4 °C.

6.1.8 Digital Analysis of the Data

The data were processed as described in U.S. Application Serial No. 08/980,574, (published as WO 98/23950) at Sections 5.4 and 5.5 (incorporated herein by reference), as set forth more particularly below.

The output from the scanner was first processed using the MELANIE® II 2D PAGE analysis program (Release 2.2, 1997, BioRad Laboratories, Hercules, California, Cat. # 170-7566) to autodetect the registration points, M1, M2, M3 and M4; to autocrop the images (*i.e.*, to eliminate signals originating from areas of the scanned image lying outside the boundaries of the gel, *e.g.* the reference frame); to filter out artifacts due to dust; to detect and quantify features; and to create image files in GIF format. Features were detected using the following parameters:

Smooths =2
Laplacian threshold 50
Partials threshold 1
Saturation = 100
Peakedness = 0
Minimum Perimeter = 10

6.1.9 Assignment of pI and MW Values

Landmark identification was used to determine the pI and MW of features detected in the images. Twelve landmark features, designated CSF1 to CSF12, were identified in a standard CSF image obtained from a pooled sample. These landmark features are identified in Figure 1 and were assigned the pI and/or MW values identified in Table XII.

Table XII. Landmark Features

Name	pI	MW (Da)	Name	pI	MW (Da)
CSF1	5.96	185230	CSF7	4.78	41340
CSF2	5.39	141700	CSF8	9.2	40000
CSF3	6.29	100730	CSF9	5.5	31900
CSF4	5.06	71270	CSF10	6.94	27440
CSF5	7.68	68370	CSF11	5.9	23990
CSF6	5.67	48090	CSF12	6.43	10960

As many of these landmarks as possible were identified in each gel image of the dataset. Each feature in the study gels was then assigned a pI value by linear interpolation or extrapolation (using the MELANIE®-II software) to the two nearest landmarks, and was assigned a MW value by linear interpolation or extrapolation (using the MELANIE®-II software) to the two nearest landmarks.

6.1.10 Matching With Primary Master Image

Images were edited to remove gross artifacts such as dust, to reject images which had gross abnormalities such as smearing of protein features, or were of too low a loading or overall image intensity to allow identification of more than the most intense features, or were of too poor a resolution to allow accurate detection of features. Images were then compared by pairing with one common image from the whole sample set. This common image, the "primary master image", was selected on the basis of protein load (maximum load consistent with maximum feature detection), a well resolved myoglobin region, (myoglobin was used as an internal standard), and general image quality. Additionally, the primary master image was chosen to be an image which appeared to be generally representative of all those to be included in the analysis. (This process by which a primary master gel was judged to be representative of the study gels was rechecked by the method described below and in the event

that the primary master gel was seen to be unrepresentative, it was rejected and the process repeated until a representative primary master gel was found.)

Each of the remaining study gel images was individually matched to the primary master image such that common protein features were paired between the primary master image and each individual study gel image as described below.

6.1.11 Cross-matching Between Samples

To facilitate statistical analysis of large numbers of samples for purposes of identifying features that are differentially expressed, the geometry of each study gel was adjusted for maximum alignment between its pattern of protein features, and that of the primary master, as follows. Each of the study gel images was individually transformed into the geometry of the primary master image using a multi-resolution warping procedure. This procedure corrects the image geometry for the distortions brought about by small changes in the physical parameters of the electrophoresis separation process from one sample to another. The observed changes are such that the distortions found are not simple geometric distortions, but rather a smooth flow, with variations at both local and global scale.

The fundamental principle in multi-resolution modeling is that smooth signals may be modeled as an evolution through 'scale space', in which details at successively finer scales are added to a low resolution approximation to obtain the high resolution signal. This type of model is applied to the flow field of vectors (defined at each pixel position on the reference image) and allows flows of arbitrary smoothness to be modeled with relatively few degrees of freedom. Each image is first reduced to a stack, or pyramid, of images derived from the initial image, but smoothed and reduced in resolution by a factor of 2 in each direction at every level (Gaussian pyramid) and a corresponding difference image is also computed at each level, representing the difference between the smoothed image and its progenitor (Laplacian pyramid). Thus the Laplacian images represent the details in the image at different scales.

To estimate the distortion between any 2 given images, a calculation was performed at level 7 in the pyramid (*i.e.* after 7 successive reductions in resolution). The Laplacian images were segmented into a grid of 16x16 pixels, with 50% overlap between adjacent grid positions in both directions, and the cross correlation between corresponding grid squares on the reference and the test images was computed. The distortion displacement was then given by the location of the maximum in the correlation matrix. After all displacements had been calculated at a particular level, they were interpolated to the next level in the pyramid, applied

to the test image, and then further corrections to the displacements were calculated at the next scale.

The warping process brought about good alignment between the common features in the primary master image, and the images for the other samples. The MELANIE® II 2D PAGE analysis program was used to calculate and record approximately 500-700 matched feature pairs between the primary master and each of the other images. The accuracy of this program was significantly enhanced by the alignment of the images in the manner described above. To improve accuracy still further, all pairings were finally examined by eye in the MelView interactive editing program and residual recognizably incorrect pairings were removed. Where the number of such recognizably incorrect pairings exceeded the overall reproducibility of the Preferred Technology (as measured by repeat analysis of the same biological sample) the gel selected to be the primary master gel was judged to be insufficiently representative of the study gels to serve as a primary master gel. In that case, the gel chosen as the primary master gel was rejected, and different gel was selected as the primary master gel, and the process was repeated.

All the images were then added together to create a composite master image, and the positions and shapes of all the gel features of all the component images were super-imposed onto this composite master as described below.

Once all the initial pairs had been computed, corrected and saved, a second pass was performed whereby the original (unwarped) images were transformed a second time to the geometry of the primary master, this time using a flow field computed by smooth interpolation of the multiple tie-points defined by the centroids of the paired gel features. A composite master image was thus generated by initialising the primary master image with its feature descriptors. As each image was transformed into the primary master geometry, it was digitally summed pixel by pixel into the composite master image, and the features that had not been paired by the procedure outlined above were likewise added to the composite master image description, with their centroids adjusted to the master geometry using the flow field correction.

The final stage of processing was applied to the composite master image and its feature descriptors, which now represent all the features from all the images in the study transformed to a common geometry. The features were grouped together into linked sets or "clusters", according to the degree of overlap between them. Each cluster was then given a unique identifying index, the molecular cluster index (MCI).

An MCI identifies a set of matched features on different images. Thus an MCI represents a protein or proteins eluting at equivalent positions in the 2D separation in different samples.

6.1.12. Construction of Profiles

After matching all component gels in the study to the final composite master image, the intensity of each feature was measured and stored. The end result of this analysis was the generation of a digital profile which contained, for each identified feature: 1) a unique identification code relative to corresponding feature within the composite master image (MCI), 2) the x, y coordinates of the features within the gel, 3) the isoelectric point (pI) of the VFs, 4) the apparent molecular weight (MW) of the VFs, 5) the signal value, 6) the standard deviation for each of the preceding measurements, and 7) a method of linking the MCI of each feature to the master gel to which this feature was matched. By virtue of a Laboratory Information Management System (LIMS), this MCI profile was traceable to the actual stored gel from which it was generated, so that proteins identified by computer analysis of gel profile databases could be retrieved. The LIMS also permitted the profile to be traced back to an original sample or patient.

6.1.13. Statistical Analysis of the Profiles

The complementary statistical strategies specified below were used in the order in which they are listed to identify VFs from the MCIs within the mastergroup.

(a) The Wilcoxon Rank-Sum test. This test was performed between the control and the Vascular Dementia samples for each MCI basis. The MCIs which recorded a p- value less than or equal to 0.05 were selected as statistically significant VFs with 95% selectivity.

(b) A second non-overlapping selection strategy is based on the fold change. A fold change representing the ratio of the average normalized protein abundances of the VFs within an MCI, was calculated for each MCI between each set of controls and Vascular Dementia samples. An 80% confidence limit for the mean of the fold changes was calculated. The MCIs with fold changes which fall outside the confidence limit were selected as VFs which met the criteria of the significant fold change threshold with 80% selectivity. Because the MCI fold changes are based on an 80% confidence limit, it follows that the significant fold change threshold is itself 80%.

(c) A third non-overlapping selection strategy is based on qualitative presence or absence alone. Using this procedure, a percentage feature presence was calculated across the

control samples and Vascular Dementia samples for each MCI which was a potential VF based on such qualitative criteria alone, *i.e.* presence or absence. The MCIs which recorded a percentage feature presence of 80% or more on Vascular Dementia samples and a percentage feature presence of 20% or less on control samples, were selected as the qualitative differential VFs with 80% selectivity. A second group of qualitative differential VFs with 80% selectivity were formed by those MCIs which recorded a percentage feature presence of 80% or more on control samples and a percentage feature presence of 20% or less on Vascular Dementia samples.

Application of these three analysis strategies allowed VFs to be selected on the basis of: (a) statistical significance as measured by the Wilcoxon Rank-Sum test, (b) a significant fold change threshold with a chosen selectivity, or (c) qualitative differences with a chosen selectivity.

6.1.14 Recovery and analysis of selected proteins

Proteins in VFs were robotically excised and processed to generate tryptic digest peptides. Tryptic peptides were analyzed by mass spectrometry using a PerSeptive Biosystems Voyager- DETM STR Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer, and selected tryptic peptides were analyzed by tandem mass spectrometry (MS/MS) using a Micromass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass, Altrincham, U.K.) equipped with a nanoflowTM electrospray Z-spray source. For partial amino acid sequencing and identification of VPIs uninterpreted tandem mass spectra of tryptic peptides were searched using the SEQUEST search program (Eng et al, *J. Am. Soc. Mass Spectrom.* (1994) 5:976-989), version v.C.1. Criteria for database identification included: the cleavage specificity of trypsin; the detection of a suite of a, b and y ions in peptides returned from the database, and a mass increment for all Cys residues to account for carbamidomethylation. The database searched was database constructed of protein entries in the non-redundant database held by the National Centre for Biotechnology Information (NCBI) which is accessible at http://www.ncbi.nlm.nih.gov/. Following identification of proteins through spectral- spectral correlation using the SEQUEST program, masses detected in MALDI-TOF mass spectra were assigned to tryptic digest peptides within the proteins identified. In cases where no amino acid sequences could be identified through searching with uninterpreted MS/MS spectra of tryptic digest peptides using the SEQUEST program, tandem mass spectra of the peptides were interpreted manually, using methods

known in the art. (In the case of interpretation of low-energy fragmentation mass spectra of peptide ions see Gaskell et al, *Rapid Commun. Mass Spectrom.* (1992) 6:658-662)

EXAMPLE: DIAGNOSIS AND TREATMENT OF VASCULAR DEMENTIA

The following example illustrates the use of a VPI of the invention for screening or diagnosis of Vascular Dementia, determining the prognosis of a Vascular Dementia patient, or monitoring the effectiveness of Vascular Dementia therapy. The following example also illustrates the use of modulators (*e.g.*, agonist or antagonists) of a VPI of the invention to treat or prevent Vascular Dementia.

Insulin-like growth factor II (IGF-II) is a pleiotropic protein belonging to the insulin family of peptide growth factors that exert trophic effects on several different cell types in the nervous system and may facilitate axonal regeneration (Hammarberg et al. J Comp Neurol (1998) 400: 57-72). Two secreted bioactive forms with a molecular size of 10 kDa and 7.5 kDa may be involved in autocrine control of cell growth. It binds to the IGF-I and IGF-II receptors on the surface of many cell types. Most of it cellular effects are mediated through the intrinsic tyrosine kinase activity of the IGF-I receptor. It biological activity can be regulated at transcriptional, translational, posttranslational processing and receptor binding level (Gammeltoft et al. Avd Exp Med Biol (1991) 293: 31-33). Furthermore, the biological effects of IGF-II its receptors are modulated by IGF-binding proteins (IGFBPs). By analogy with other transport proteins synthesized by the choroid plexus, IGFBPs may facilitate the secretion of IGF-II to the cerebrospinal fluid and modulate its biological actions at distant sites within the brain. IGFBPs bind IGFs with equal or higher affinity than the IGF-I receptor suggesting that one of their roles is to sequester free IGFs. IGFs may be released through proteolytic cleavage of IGFBPs by aspartic, serine and Matrix Metalloproteinases (MMPs) (Conover and De Leon J Biol Chem (1994) 269:7076-7080; Fowlkes et al. J Biol Chem 269, 25742-25746), since IGFBP fragments have a lower affinity to IGFs and intact IGFBPs (Lassare and Binoux Endocrinology (1994) 134:1254-1262). The proteolytic activity of MMPs are precisely regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Therefore, the balance between TIMPs and MMPs may regulate IGF bioavailability. This has been demonstrated for TIMP-1 and IGF-II in a transgenic rodent model, whereas overexpresssion of TIMP-1 blocks IGF-II signaling in vivo (Martin et al. The Journal of Cell Biology (1999) 146:881-892).

Recent evidence from experimental rodent models suggest, that under normal physiology, IGF-II is mainly expressed in mesenchymal support structures of the brain, such

as the choroid plexus, where its expression is coincides with that of IGFBP-2. During the acute phase of a brain injury (within the first 7 days), secretion from the choroid plexus is up-regulated and subsequently sequestered through increased levels of IGFBP-5. In the chronic phase of the injury IGF-II expression may be restricted to the site of injury via local expression in marginal astrocytes in a predominantly autocrine/paracrine fashion. (Walter et al.,1999, Endocrinology 140:520-32). The rather unique predisposition of IGF-II to act on a selection of neuronal cells may offer a general means of reducing and/or slowing down neuronal losses that occur following various brain insults.

The expression of TIMP-I with a molecular weight of 23,795Da and a pI of 9.80 has been shown to be significantly decreased in the cerebrospinal fluid (CSF) of subjects having vascular dementia as compared with the CSF of subjects free from vascular dementia and the expression of IGF-II with a molecular weight of 10,226Da and a pI of 6.53 has been shown herein to be significantly increased in the cerebrospinal fluid (CSF) of subjects having vascular dementia as compared with the CSF of subjects free from vascular dementia (see Tables I and II). Thus, quantitative detection of TIMP-I and/or IGF-II in CSF can be used to diagnose vascular dementia, determine the progression of vascular dementia or monitor the effectiveness of a therapy for vascular dementia.

In one embodiment of the invention, compounds that modulate (*e.g.*, upregulate or downregulate) the expression, activity or both the expression and activity of IGF-II are administered to a subject in need of treatment or for prophylaxis of Vascular dementia. Antibodies that modulate the expression, activity or both the expression and activity of IGF-II are suitable for this purpose. In addition, nucleic acids coding for all or a portion of IGF-II, or nucleic acids complementary to all or a portion of IGF-II, may be administered. IGF-II, or fragments of the IGF-II polypeptide may also be administered.

The invention also provides screening assays to identify additional compounds that modulate the expression of IGF-II or activity of IGF-II. Compounds that modulate the expression of IGF-II in vitro can be identified by comparing the expression of IGF-II in cells treated with a test compound to the expression of IGF-II in cells treated with a control compound (*e.g.*, saline). Methods for detecting expression of IGF-II are known in the art and include measuring the level of IGF-II RNA (*e.g.*, by northern blot analysis or RT-PCR) and measuring IGF-II protein (*e.g.*, by immunoassay or western blot analysis). Compounds that modulate the activity of IGF-II can be identified by comparing the ability of a test compound to agonize or antagonize a function of IGF-II, such as its neurotrophic activity or its binding to the IGF-I receptor, to the ability of a control compound (*e.g.*, saline) to inhibit the same

function of IGF-II. Compounds capable of modulating IGF-II binding to its receptor or IGF-II activity are identified as compounds suitable for further development as a compound useful for the treatment of vascular dementia.

Binding between IGF-II and its receptor can be determined by, for example, contacting IGF-II with cells known to express the IGF-II receptor and assaying the extent of binding between IGF-II and the cell surface receptor, or by contacting IGF-II with its receptor in a cell-free assay, *i.e.*, an assay where the IGF-II and IGF-II receptor are isolated, and, preferably, recombinantly produced, and assaying the extent of binding between IGF-II and its receptor. Through the use of such assays, candidate compounds may be tested for their ability to agonize or antagonize the binding of IGF-II to its receptor.

Compounds identified *in vitro* that affect the expression or activity of IGF-II can be tested *in vivo* in animal models of vascular dementia, or in subjects having vascular dementia, to determine their therapeutic efficacy.

The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The contents of each reference, patent and patent application cited in this application is hereby incorporated by reference in its entirety.

WE CLAIM:

1. A method for screening, diagnosis or prognosis of Vascular Dementia in a subject, for determining the stage or severity of Vascular Dementia in a subject, for identifying a subject at risk of developing Vascular Dementia, or for monitoring the effect of therapy administered to a subject having Vascular Dementia, said method comprising:

(a) analyzing a test sample of body fluid from the subject by two dimensional electrophoresis to generate a two-dimensional array of features, said array comprising at least one chosen feature whose relative abundance correlates with the presence, absence, stage or severity of Vascular Dementia or predicts the onset or course of Vascular Dementia; and

(b) comparing the abundance of each chosen feature in the test sample with the abundance of that chosen feature in body fluid from one or more persons free from Vascular Dementia, or with a previously determined reference range for that feature in subjects free from Vascular Dementia, or with the abundance at least one Expression Reference Feature (ERF) in the test sample.

2. The method of claim 1, wherein the body fluid is cerebrospinal fluid (CSF).

3. The method of claim 1 or claim 2, wherein said method is for screening or diagnosis of Vascular Dementia and the relative abundance of at least one chosen feature correlates with the presence or absence of Vascular Dementia.

4. The method of claim 1 or claim 2, wherein said method is for monitoring the effect of therapy administered to a subject having Vascular Dementia and the relative abundance of at least one chosen feature correlates with the severity of Vascular Dementia.

5. The method of claim 2, wherein step (b) comprises comparing the abundance of each chosen feature in the sample with the abundance of that chosen feature in CSF from one or more persons free from Vascular Dementia or with a previously determined reference range for that chosen feature in subjects free from Vascular Dementia.

6. The method of claim 1 or claim 2, wherein step (b) comprises detecting one or more of the following Vascular Dementia-Associated Features (VFs): VF-4, VF-5, VF-12, VF-13, VF-14, VF-15, VF-16, VF-17, VF-18, VF-19, VF-20, VF-21, VF-22, VF-23, VF-24,

VF-25, VF-26, VF-27, VF-29, VF-30, VF-31, VF-32, VF-33, VF-34, VF-35, VF-36, VF-37, VF-38, VF-41, VF-42, VF-43, VF-44, VF-45, VF-46, VF-47, VF-48, VF-50, VF-51, VF-52, VF-53, VF-54, VF-55, VF-57, VF-58, VF-60, VF-64, VF-66, VF-68, VF-92, VF-93, VF-94, VF-95, VF-96, VF-97, VF-98, VF-99, VF-100, VF-101, VF-102, VF-103, VF-104, VF-105, VF-106, VF-107, VF-108, VF-109, VF-110, VF-111, VF-112, VF-113, VF-114, VF-115, VF-116, VF-118, VF-120, VF-121, VF-122, VF-123, VF-124, VF-125, VF-126, VF-127, VF-128, VF-129, VF-130, VF-131, VF-132, VF-134, VF-135, VF-136, VF-137, VF-138, VF-139, VF-140, VF-141, VF-142, VF-143, VF-144, VF-145, VF-146, VF-147, VF-148, VF-149, VF-150, VF-151, VF-152, VF-153, VF-154, VF-155, VF-156, VF-157, VF-158, VF-159, VF-160, VF-161, VF-162, VF-163, VF-164, VF-166, VF-168, VF-170, VF-171, VF-172, VF-173, VF-174, VF-175, VF-176, VF-177, VF-178, VF-179, VF-180, VF-181, VF-182, VF-183, VF-184, VF-185, VF-186, VF-187, VF-188, VF-189, VF-190, VF-191, VF-192, VF-193, VF-194, VF-195, VF-196, VF-197, VF-198, VF-199, VF-200, VF-201, VF-202, VF-203, VF-204, VF-205, VF-206, VF-207, VF-208, VF-209, VF-210, VF-211, VF-212, VF-213, VF-214, VF-215, VF-216, VF-217, VF-218, VF-219, VF-220, VF-221, VF-222, VF-223, VF-224, VF-225, VF-226, VF-227, VF-228, VF-229, VF-230, VF-231, VF-232, VF-233, VF-234, VF-235, VF-236, VF-237, VF-238, VF-239, VF-240, VF-241, VF-242, VF-243, VF-244, VF-245, VF-246, VF-247, VF-248, VF-249, VF-250, VF-251, VF-252, VF-253, VF-254, VF-255, VF-256, VF-257, VF-258, VF-259, VF-260, VF-261, VF-262, VF-263, VF-264, VF-265, VF-266, VF-267, VF-268, VF-269, VF-270, VF-271, VF-272, VF-273, VF-274, VF-275, VF-276, VF-277, VF-278, VF-279, VF-280, VF-281, VF-282, VF-283, VF-284, VF-285, VF-286, VF-287, VF-288, VF-289, VF-290, VF-291, VF-292, VF-293, VF-294, VF-295, VF-296, VF-297, VF-298, VF-299, VF-300, VF-301, VF-302, VF-303, VF-304, VF-305, VF-306, VF-307, VF-308, VF-309, VF-310, VF-311, VF-312, VF-313, VF-314, VF-315, VF-316, VF-317, VF-318, VF-319, VF-320, VF-321, VF-322, VF-323, VF-324, VF-325, VF-326, VF-327, VF-328, VF-329, VF-330, VF-331, VF-332, VF-333, VF-334, VF-335, VF-336, VF-337, VF-338, VF-339, VF-340, VF-341, VF-342, VF-343, VF-344, VF-345, VF-346, VF-347, VF-348, VF-349, VF-350, VF-351, VF-352, VF-353, VF-354, VF-355, VF-356, VF-357, VF-358, VF-359, VF-360, VF-361, VF-362, VF-363, VF-364, VF-365, VF-366, VF-367, VF-368, VF-369, VF-370, VF-371, or VF-372.

7. The method according to claim 1, 2, or 5, wherein step (a) comprises isoelectric focussing followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

8. A method for screening, diagnosis or prognosis of Vascular Dementia in a subject, for determining the stage or severity of Vascular Dementia in a subject, for identifying a subject at risk of developing Vascular Dementia, or for monitoring the effect of therapy administered to a subject having Vascular Dementia, said method comprising detecting, in a sample of cerebrospinal fluid from the subject, at least one of the following Vascular Dementia-Associated Protein Isoforms (VPIs): VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273.

9. The method according to claim 8, wherein the step of detecting comprises testing at least one aliquot of the sample, said step of testing comprising:

(a) contacting the aliquot with an antibody that is immunospecific for a preselected VPI; and

(b) measuring any binding that has occurred between the antibody and at least one species in the aliquot.

10. The method according to claim 9, wherein the antibody is a monoclonal antibody.

11. The method according to claim 9, wherein the step of detecting comprises testing a plurality of aliquots with a plurality of antibodies for detection of a plurality of preselected VPIs.

12. The method according to claim 11, wherein the antibodies are monoclonal antibodies.

13. A preparation comprising one of the following isolated Vascular Dementia-Associated Protein Isoforms (VPIs): VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-

248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273.

14. A kit comprising the preparation of claim 13.

15. A kit comprising a plurality of distinct preparations of claim 14.

16. An antibody capable of immunospecific binding to one of the following Vascular Dementia-Associated Protein Isoforms (VPIs): VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273.

17. The antibody of claim 16, which is a monoclonal antibody.

18. The antibody of claim 16 or 17, which binds to the VPI with greater affinity than to another isoform of the VPI.

19. The antibody of claim 16, which binds to the VPI with greater affinity than to any other isoform of the VPI.

20. A kit comprising the antibody of claim 16.

21. A kit comprising a plurality of distinct antibodies of claim 16.

22. A pharmaceutical composition comprising a therapeutically effective amount of an antibody of claim 16 and a pharmaceutically acceptable carrier.

23. A pharmaceutical composition comprising: a therapeutically effective amount of a fragment or derivative of an antibody of claim 16, said fragment or derivative containing the binding domain of the antibody; and a pharmaceutically acceptable carrier.

24. A method of treating or preventing Vascular Dementia comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid encoding one of the following Vascular Dementia- Associated Protein Isoforms (VPIs): VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-

183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273.

25. A method of treating or preventing Vascular Dementia comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid that inhibits the function of one or more of the following Vascular Dementia-Associated Protein Isoforms (VPIs): VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246,

VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273.

26. The method of claim 25, wherein the nucleic acid is a VPI antisense nucleic acid or ribozyme.

27. A method of screening for agents that interact with a VPI, a VPI fragment, or a VPI-related polypeptide, said method comprising:

(a) contacting a VPI, a biologically active portion of a VPI, or a VPI-related polypeptide with a candidate agent; and

(b) determining whether or not the candidate agent interacts with the VPI, the VPI fragment, or the VPI-related polypeptide.

28. The method of claim 27, wherein the VPI, the VPI fragment, or the VPI-related polypeptide is expressed by cells.

29. The method of claim 27, wherein the cells express a recombinant VPI, a recombinant VPI fragment, or a recombinant VPI-related polypeptide.

30. A method of screening for agents that modulate the expression or activity of a VPI or a VPI-related polypeptide comprising:

(a) contacting a first population of cells expressing a VPI or a VPI-related polypeptide with a candidate agent;

(b) contacting a second population of cells expressing said VPI or said VPI-related polypeptide with a control agent; and

(c) comparing the level of said VPI or said VPI-related polypeptide or mRNA encoding said VPI or said VPI-related polypeptide in the first and second populations of cells, or comparing the level of induction of a cellular second messenger in the first and second populations of cells.

31. The method of claim 30, wherein the level of said VPI or said VPI- related polypeptide, mRNA encoding said VPI or said VPI-related polypeptide, or said cellular

second messenger is greater in the first population of cells than in the second population of cells.

32. The method of claim 30, wherein the level of said VPI or said VPI- related polypeptide, mRNA encoding said VPI or said VPI-related polypeptide, or said cellular second messenger is less in the first population of cells than in the second population of cells.

33. A method of screening for or identifying agents that modulate the expression or activity of a VPI or a VPI-related polypeptide comprising:

(a) administering a candidate agent to a first mammal or group of mammals;

(b) administering a control agent to a second mammal or group of mammals; and

(c) comparing the level of expression of the VPI or the VPI-related polypeptide or of mRNA encoding the VPI or the VPI-related polypeptide in the first and second groups, or comparing the level of induction of a cellular second messenger in the first and second groups.

34. The method of claim 33, wherein the mammals are animal models for Vascular Dementia.

35. The method of claim 33 or 34, wherein the level of expression of said VPI or said VPI-related polypeptide, mRNA encoding said VPI or said VPI-related polypeptide, or of said cellular second messenger is greater in the first group than in the second group.

36. The method of claim 33 or 34, wherein the level of expression of said VPI or said VPI-related polypeptide, mRNA encoding said VPI or said VPI-related polypeptide, or of said cellular second messenger is less than in the first group than in the second group.

37. The method of claim 33, wherein the levels of said VPI or said VPI- related polypeptide, mRNA encoding said VPI or said VPI-related polypeptide, or of said cellular second messenger in the first and second groups are further compared to the level of said VPI or said VPI-related polypeptide or said mRNA encoding said VPI or said VPI-related polypeptide in normal control mammals.

38. The method of claim 33, wherein administration of said candidate agent modulates the level of said VPI or said VPI-related polypeptide, or said mRNA encoding said VPI or said VPI-related polypeptide, or said cellular second messenger in the first group towards the levels of said VPI or said VPI-related polypeptide or said mRNA or said cellular second messenger in the second group.

39. The method of claim 33, wherein said mammals are human subjects having Vascular Dementia.

40. A method of screening for or identifying agents that interact with a VPI or a VPI-related polypeptide, comprising (a) contacting a candidate agent with the VPI or the VPI-related polypeptide, and (b) detecting binding, if any, between the agent and the VPI or the VPI-related polypeptide.

41. A method of screening for or identifying agents that modulate the activity of a VPI or a VPI-related polypeptide, comprising: (a) in a first aliquot, contacting a candidate agent with the VPI or the VPI-related polypeptide, and (b) comparing the activity of the VPI or the VPI-related polypeptide in the first aliquot after addition of the candidate agent with the activity of the VPI or the VPI-related polypeptide in a control aliquot, or with a previously determined reference range.

42. The method according to claim 40 or 41, wherein the VPI or the VPI- related polypeptide is recombinant protein.

43. The method according to claim 40 or 41, wherein the VPI or the VPI- related polypeptide is immobilized on a solid phase.

44. A method for screening, diagnosis or prognosis of Vascular Dementia in a subject or for monitoring the effect of an anti-Vascular Dementia drug or therapy administered to a subject, comprising:

(a) contacting at least one oligonucleotide probe comprising 10 or more consecutive nucleotides complementary to a nucleotide sequence encoding a VPI chosen from VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-

31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63, VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273 with an RNA obtained from a biological sample from the subject or with cDNA copied from the RNA wherein said contacting occurs under conditions that permit hybridization of the probe to the nucleotide sequence if present;

(b) detecting hybridization, if any, between the probe and the nucleotide sequence; and

(c) comparing the hybridization, if any, detected in step (b) with the hybridization detected in a control sample, or with a previously determined reference range.

45. The method of claim 44, wherein step (a) comprises contacting a plurality of oligonucleotide probes comprising 10 or more consecutive nucleotides complementary to a nucleotide sequence encoding a VPI chosen from VPI-2, VPI-3, VPI-6, VPI-7, VPI-8, VPI-9, VPI-10, VPI-11, VPI-13, VPI-14, VPI-15, VPI-17, VPI-18, VPI-19, VPI-21, VPI-22, VPI-23, VPI-24, VPI-25, VPI-27, VPI-28, VPI-29, VPI-31, VPI-32, VPI-33, VPI-37, VPI-38, VPI-40, VPI-43, VPI-46, VPI-48, VPI-49, VPI-50, VPI-56, VPI-57, VPI-58, VPI-61, VPI-62, VPI-63,

VPI-64, VPI-65, VPI-66, VPI-67, VPI-68, VPI-69, VPI-70, VPI-71, VPI-72, VPI-73, VPI-75, VPI-77, VPI-78, VPI-79, VPI-80, VPI-81, VPI-82, VPI-83, VPI-84, VPI-85, VPI-87, VPI-88, VPI-90, VPI-91, VPI-92, VPI-93, VPI-94, VPI-95, VPI-96, VPI-97, VPI-98, VPI-99, VPI-100, VPI-108, VPI-109, VPI-110, VPI-111, VPI-112, VPI-113, VPI-114, VPI-115, VPI-116, VPI-117, VPI-118, VPI-119, VPI-120, VPI-121, VPI-122, VPI-123, VPI-124, VPI-125, VPI-127, VPI-128, VPI-129, VPI-130, VPI-131, VPI-133, VPI-146, VPI-147, VPI-148, VPI-149, VPI-150, VPI-152, VPI-153, VPI-154, VPI-155, VPI-156, VPI-158, VPI-159, VPI-160, VPI-161, VPI-162, VPI-163, VPI-164, VPI-165, VPI-166, VPI-167, VPI-168, VPI-169, VPI-170, VPI-171, VPI-172, VPI-173, VPI-174, VPI-175, VPI-176, VPI-177, VPI-178, VPI-179, VPI-180, VPI-181, VPI-182, VPI-183, VPI-184, VPI-185, VPI-186, VPI-187, VPI-188, VPI-189, VPI-190, VPI-191, VPI-192, VPI-193, VPI-194, VPI-195, VPI-196, VPI-197, VPI-198, VPI-199, VPI-200, VPI-201, VPI-202, VPI-203, VPI-204, VPI-205, VPI-206, VPI-207, VPI-208, VPI-209, VPI-210, VPI-211, VPI-212, VPI-213, VPI-214, VPI-215, VPI-216, VPI-217, VPI-218, VPI-219, VPI-220, VPI-221, VPI-222, VPI-223, VPI-224, VPI-225, VPI-226, VPI-227, VPI-228, VPI-229, VPI-230, VPI-231, VPI-232, VPI-233, VPI-234, VPI-235, VPI-236, VPI-237, VPI-238, VPI-239, VPI-240, VPI-241, VPI-242, VPI-243, VPI-244, VPI-245, VPI-246, VPI-247, VPI-248, VPI-249, VPI-250, VPI-251, VPI-252, VPI-253, VPI-254, VPI-255, VPI-256, VPI-257, VPI-258, VPI-259, VPI-260, VPI-261, VPI-262, VPI-263, VPI-264, VPI-265, VPI-266, VPI-267, VPI-268, VPI-269, VPI-270, VPI-271, VPI-272, or VPI-273 with an RNA obtained from a biological sample from the subject or with cDNA copied from the RNA wherein said contacting occurs under conditions that permit hybridization of the probe to the nucleotide sequence if present.

46. The method of claim 44, wherein step (a) includes the step of hybridizing the nucleotide sequence to a DNA array, wherein one or more members of the array are the probes complementary to a plurality of nucleotide sequences encoding distinct VPIs.

47. A method of screening for agents effective for the treatment of vascular dementia comprising:

(1) contacting IGF-II with a first population of cells expressing a receptor for IGF-II in the presence of a candidate agent;

(2) contacting IGF-II with a second population of cells expressing said receptor in the presence of a control agent;

(3) comparing the binding of said IGF-II to the first and second populations of cells, or comparing the level of induction of a cellular second messenger in the first and second populations of cells, or comparing the level of a IGF-II mediated activity in the first and second population of cells; and

(4) testing for the ability of agents able to modulate the activity of IGF-II to decrease clinical features of vascular dementia in an vascular dementia disease model system.

48. The method of claim 47 wherein the IGF-II or the receptor, or both, are isolated and recombinantly produced.

49. The method of claim 47, wherein the cells are cerebellar granule cells and the IGF-II mediated activity is protection of immature cerebellar cells from apoptotic death.

50. The method of claim 47, wherein the cells are SY5Y cells or cerebellar granule cells and the binding of IGF-II to the cells is compared.

51. The method of claim 47, wherein the candidate agent comprises at least 10 consecutive amino acids of IGF-II.

52. A method of screening for agents that modulate the binding of IGF-II to a binding partner comprising:

(1) contacting IGF-II with the IGF-II binding partner in the presence of a candidate agent;

(2) contacting IGF-II with a IGF-II binding partner in the presence of a control agent; and

(3) comparing the binding of said IGF-II to the binding partner in step (a) to the binding of said IGF-II to the binding partner in step (b).

53. The method of claim 52 wherein the binding partner is an isolated IGF-I receptor.

54. The method of claim 53 wherein the IGF-II and the IGF-I receptor are recombinantly produced.

55. A method of screening for agents effective for the treatment of vascular dementia comprising:

(1) contacting TIMP-1 with a first population of cells expressing MMPs in the presence of a candidate agent;

(2) contacting TIMP-1 with a second population of cells expressing MMPs in the presence of a control agent;

(3) comparing the binding of said TIMP-1 to the first and second populations of cells, or comparing the level of induction of a cellular second messenger in the first and second populations of cells, or comparing the level of a TIMP-1 mediated activity in the first and second population of cells; and

(4) testing for the ability of agents able to modulate the activity of TIMP-1 to decrease clinical features of vascular dementia in an vascular dementia disease model system.

56. The method of claim 55 wherein the TIMP-1 or the receptor, or both, are isolated and recombinantly produced.

57. The method of claim 55, wherein the cells are cerebellar granule cells and the TIMP-1 mediated activity is protection of immature cerebellar cells from apoptotic death.

58. The method of claim 55, wherein the cells are SY5Y cells or cerebellar granule cells and the binding of TIMP-1 to the cells is compared.

59. The method of claim 55, wherein the candidate agent comprises at least 10 consecutive amino acids of TIMP-1.

60. A method of screening for agents that modulate the binding of TIMP-1 to a binding partner comprising:

(1) contacting TIMP-1 with the TIMP-1 binding partner in the presence of a candidate agent;

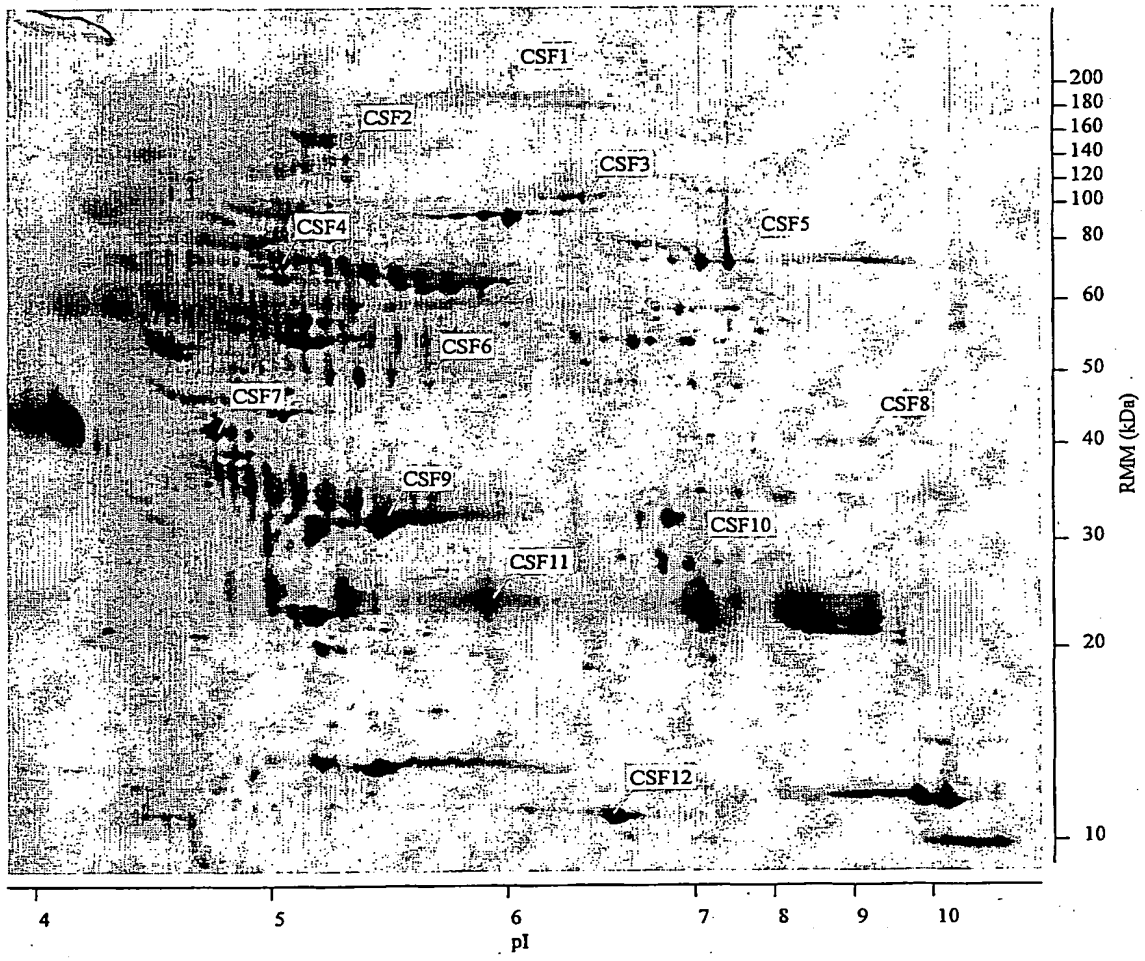
(2) contacting TIMP-1 with a TIMP-1 binding partner in the presence of a control agent; and

(3) comparing the binding of said TIMP-1 to the binding partner in step (a) to the binding of said TIMP-1 to the binding partner in step (b).

61. The method of claim 60 wherein the binding partner is an isolated MMP receptor.

62. The method of claim 61 wherein the TIMP-1 and the MMP receptor are recombinantly produced.

FIGURE 1


CSF1
CSF2
CSF3
CSF4
CSF5
CSF6
CSF7
CSF8
CSF9
CSF10
CSF11
CSF12
200
180
160
140
120
100
80
60
50
40
30
20
10
RMM (kDa)
4
5
6
7
8
9
10
pI